EXHIBIT 4

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12P 17/02, A61K 35/78	A1	(11) International Publication Number: WO 93/02204
		(43) International Publication Date: 4 February 1993 (04.02.93)

(21) International Application Number: PCT/KR92/00031

(22) International Filing Date: 16 July 1992 (16.07.92)

(30) Priority data:
91-12268 18 July 1991 (18.07.91) KR

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(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE).

Published
With international search report.

(54) Title: PRODUCTION OF GINKGOLIDES IN CELL CULTURE

(57) Abstract

Tissues of *Ginkgo biloba* have been successfully cultured to produce chemotherapeutically active diterpene compounds, ginkgolides (especially ginkgolides A and B). Ginkgolides can be recovered from the resultant callus and suspension cultured cells and culture medium via extraction. These procedures will provide a supply of chemotherapeutic agents.

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PRODUCTION OF GINKGOLIDES IN CELL CULTURE

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the production and recovery of diterpene compounds, ginkgolides known to be antagonists of platelet activating factor (PAF) by cell culture of the tissues of the *Ginkgo biloba* (Ginkgoaceae).

Description of Prior Art

The fan-shaped bilobate leaves of *Ginkgo biloba* which are used for medicinal purposes, contain a complex mixture of original and characteristic flavonoids in free and glycosidated forms, unique terpene derivatives and other minor substances that contribute in synergistic manner to the multifarious activity. of *Ginkgo biloba*(Boralle, N., Braquet, P. and Gottlieb, O. R. Ginkgo biloba: A review of its chemical composition In: *Ginkgolides- Chemistry*, Biology, Pharmacology and Clinical Perspectives. P. Braquet (Ed.) J. R. Prous Science Publisher pp 9-25 (1988) , Schwabe, W. In : *Tebonin, Tebonin forte.*(Ed.) Schwabe Co., Karlsrube, pp7-9 (1986), Drieu K., La Presse Medicale, 31, 1455-1457 (1986)).

As far as the diterpene derivatives are concerned, a very important and particular class of diterpenes, namely ginkgolides A, B, C, J, and M, which are today considered very promising agents for the treatment of asthmatic and allergic diseases and blood rheology have been isolated (Braquet, P., The ginkgolides: Potent platelet-activating factor antagonists isolated from *Ginkgo biloba* L. : Chemistry, Pharmacology and clinical applications. *Drugs of the future* 12, 643-699 (1987)). Among the five ginkgolides, ginkgolide B has been found to exert the most potent PAF

antagonistic activity.

For continued testing of the ginkgolides as a promising agent and subsequent application as a commercial product, large quantities of the ginkgolides are required. However, the obtaining of the large amounts of ginkgolides from the Ginkgo leaves may bear the problems because of their small contents in the leaves(Okebe, K., Yamada, K., Yamamura, S. and Takada, S., Ginkgolides, *J. Chem. Soc.*, 21, 2201-2206 (1967), Nakanishi, K. The ginkgolides, *Pure and Applied Chemistry* 14, 89-113 (1967)). Many attempts have been made to obtain ginkgolides by alternative ways. The total synthesis of ginkgolide B was accomplished by means of an elegant and sophisticated pathway (Corey E. J., Kang M.C., Desai M. C., Ghosh, A. K. and Houpis, I. N., Total synthesis of (±)-ginkgolide B, *J. Am. Chem. Soc.*, 110, 649-651 (1988)). However, the successful synthesis of ginkgolide B in a small laboratory scale could not be extrapolated to a large industrial scale. Therefore, the natural sources still remain as the sole way to obtain these compounds for pharmacological and clinical experimentations at the present time. However, the *G. biloba* tree grows slowly and ten thousand pounds of leaves are generally required to produce one pound of ginkgolides. Moreover, the contents of ginkgolides depends on the location, climate and seasons collected. One promising way to resolve the problem for supplying the ginkgolides is the mass culture of *Ginkgo biloba* cells. Therefore, biotechnology holds the promise of obtaining ginkgolides in commercially viable quantities.

SUMMARY OF THE INVENTION

We have now discovered that dedifferentiated or callus cells from *G. biloba* tissues can be sucessfully grown on artificial media and that the same chemotherapeutically active ginkgolides are produced in culture as in the intact plant. Previous studies indicate that, in many cases, it is generally believed that plants do not produce the same compounds in culture as in intact plants

(Benjamin et al., *Planta Medica* 23 :394-397 (1973)). Our method for the production of ginkgolides comprises the following steps:

1. providing living tissue of *Ginkgo biloba*;
2. providing a nutrient culture medium suitable for callus formation from said tissue and for suspension cell growth;
3. culturing said tissue on said medium to induce callus from said tissue and for callus and suspension cell growth;
4. recovery of ginkgolides from said callus and suspension cells and from said medium.

Optionally, a precursor or an inducer may be added to step (3), thereby optimizing the production of ginkgolides.

Finding ginkgolides in the cell cultures was unexpected insofar as they are normally found in the leaves and roots bark. Production of ginkgolides by cell culture assures an adequate supply of the compounds as a promising drug. The ginkgolides were found in the culture supernatant and were easily extracted with ethylacetate and ether. This simple recovery and extraction as compared to processing tree leaves will be an additional advantage in commercial production.

It is therefore an object of this invention to grow cells from *G. biloba* tissues in callus and suspension culture, and in further scale-up cultures.

It is also an object of the invention to produce chemotherapeutically active ginkgolides, especially ginkgolides A and B from the callus tissue or in cells as well as in the culture medium.

Another object of the invention is to recover ginkgolides from the culture medium as well as from the callus tissue or cells.

Other objects and advantages of the invention will become readily apparent from the ensuing description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be described in greater detail with reference to the drawings in which :

FIGURE 1 is photographs showing the effect of the types and concentrations of auxins on the induction of callus from the leaves of *Ginkgo biloba* ;

FIGURE 2 is photographs showing the effect of various combinations of NAA and kinetin or BA on the growth of callus derived from the leaves of *Ginkgo biloba* under the dark or light ;

FIGURE 3 is photographs showing the effect of the different types of culture medium on the induction of callus derived from the leaves of *Ginkgo biloba* ;

FIGURE 4 is photographs showing the effect of the different types of culture medium on the growth of callus derived from the leaves of *Ginkgo biloba* ;

A : MS, B : B-5, C : ER, D : W, E : And, F : N_6

G : N-N, H : SH

FIGURE 5 is a graph showing the time course of the cell growth in suspension cultures of *Ginkgo biloba* ;

FIGURE 6 is a photograph showing the effect of activated charcoal on the development of the roots of *Ginkgo biloba* regenerated by the embryo culture ;

FIGURE 7 is a photograph showing the induction of callus from the roots of *Ginkgo biloba* regenerated by the embryo culture ;

FIGURE 8-1A is GC-MS spectrums of authentic ginkgolide A ;

FIGURE 8-1B is GC-MS spectrums of authentic ginkgolide B ;

FIGURE 8-1C is GC-MS spectrums of authentic ginkgolide C ;

FIGURE 8-2A is GC-MS spectrums of the extract of the callus derived from *Ginkgo biloba* leaves, wherein the arrow indicates the peak of ginkgolide A ;

FIGURE 8-2B is GC-MS spectrums of the extract of the callus derived from *Ginkgo biloba* leaves, wherein the arrow indicates the peak of ginkgolide B ;

FIGURE 8-2C is GC-MS spectrums of the extract of the callus derived from *Ginkgo biloba* leaves, wherein the arrow indicates the peak of ginkgolide C ;

FIGURE 9-1 is a Gas chromatogram of authentic ginkgolides A (GKA), B (GKB) and C(GKC) ;

FIGURE 9-2 is a Gas chromatogram of the extract of the callus derived from the leaves of *Ginkgo biloba* ;

FIGURE 9-3 is Gas chromatograms of the extract of the suspension cultured cells derived from the roots of *Ginkgo biloba*, comprising A : Gas chromatogram of the extract of the suspension cultured cells derived from the roots of *Ginkgo biloba*, B : Gas chromatogram of authentic ginkgolide B and C : Gas chromatogram of the co-injected extract of the suspension cultured cells derived from the roots of *Ginkgo biloba* and authentic ginkgolide B;

FIGURE 9-4 is a Gas chromatogram of the extract of the suspension cultured medium, wherein the arrow indicates the peaks of GKA and GKB ;

FIGURE 9-5 is a Gas chromatogram of the extract of the suspension cultured medium,wherein the arrow indicates the peaks of GKA, GKB and GKC with the extract of the suspension cultured medium ;

FIGURE 10-1 is a HPLC chromatogram of authentic ginkgolides (GKA), B(GKB) and C(GKC) ;

FIGURE 10-2 ia a HPLC chromatogram of the extract of the callus derived from the leaves of *Ginkgo biloba*.

Detailed Description of the Invention

The plant material of this invention is obtained from the *Ginkgo biloba*. Tissue from any part of the plant, including the leaves, stem, roots and embryos may be selected for inducing callus. However, for optimum yield of ginkgolides, leaf or root tissue is preferred to.

To prevent contamination of the culture, the tissue should be surface-sterilized prior to introducing it into the culture medium. Any conventional sterilization technique, such as chlorinated bleach treatment would normally be effective.

Under appropriate conditions, plant tissue cells may undergo dedifferentiation, i.e., change to precursor cells and form a tissue known as callus. Dedifferentiated cells or callus may be grown either as solid tissue or, preferably, as a cell suspension of single cells or small groups of cells in a culture medium. Metabolic products of the callus and suspension cultured cells, such as ginkgolides, may be isolated from the callus, suspension cultured cells or the culture medium.

A suitable culture medium for callus induction and subsequent growth is an aqueous or agar solidified medium of Murashige and Skoog's (MS) supplemented with ingredients described in Tables I and II.

Table I Formulation of Murashige and Skoog medium

Compound	mg/l	Compound	mg/l
NH_4NO_3	1650.00	KNO_3	1900.00
$CaCl_2 \cdot 2H_2O$	440.00	$MgSO_4 \cdot 7H_2O$	370.00
KH_2PO_4	170.00	$Na_2 \cdot EDTA$	37.30
$FeSO_4 \cdot 7H_2O$	27.80	$MnSO_4 \cdot 4H_2O$	22.30
$ZnSO_4 \cdot 7H_2O$	8.60	H_3BO_3	6.20
KI	0.83	$Na_2MoO_4 \cdot 2H_2O$	0.25
$CoCl_2 \cdot 6H_2O$	0.025	$CuSO_4 \cdot 5H_2O$	0.025

Table II Ingredients of the supplementation of MS medium

Compound	Amount/liter
Sucrose	30.0 g
Nicotinic Acid	0.5 mg
Pyridoxin-HCl	0.5 mg
myo-Inositol	100.0 mg
Thiamine· HCl	0.1 mg
Glycine	2.0 mg
1-Naphthalenacetic Acid	1.0 mg
Kinetin	0.1 mg

It is understood that modifications may be made in this medium such as substitution of other conventional salt compositions (e.g., Anderson's or Schenk and Hildebrandt), addition or deletion of various components, or alteration of proportions. Thus, it is apparent that determination of suitable and optimum media for induction and growth of callus would be within the ability of a person skilled in the art.

The medium may be gelled with agar for callus induction and subsequent growth, preferably in an amount of 0.8-1.0%.

Temperatures ranging from 24 to 26° C are preferable for inducing and growing the cell cultures, but higher or lower temperatures than above mentioned temperature could also be used.

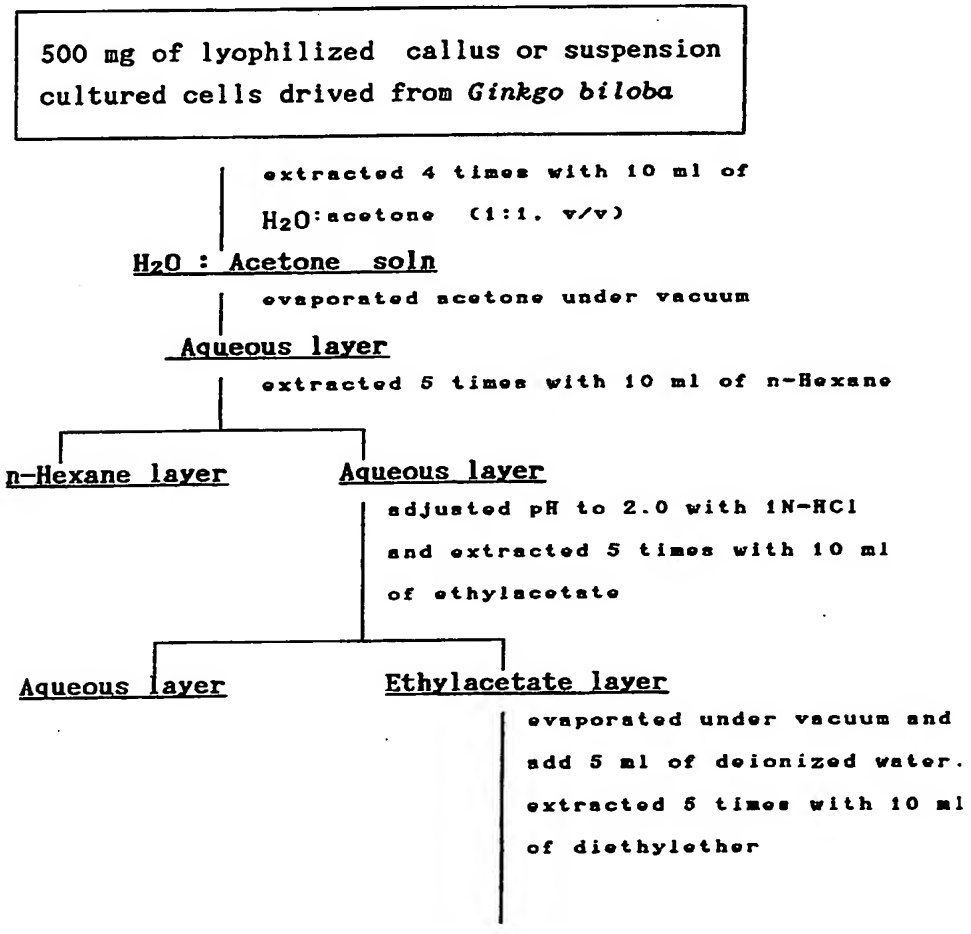
Darkness is preferred for the induction of callus. But, light is preferred for the growth of callus and suspension cultured cells. Generally, 2 to 4 weeks are required for the callus induction from plant tissue. Callus cultures on agar plates are subcultured at 3-week interval. Callus growth and ginkgolides production can be revitalized by subculturing, in which a portion of the callus is transferred to fresh media. The cells that are grown in suspension culture are subcultured at 3-week interval,

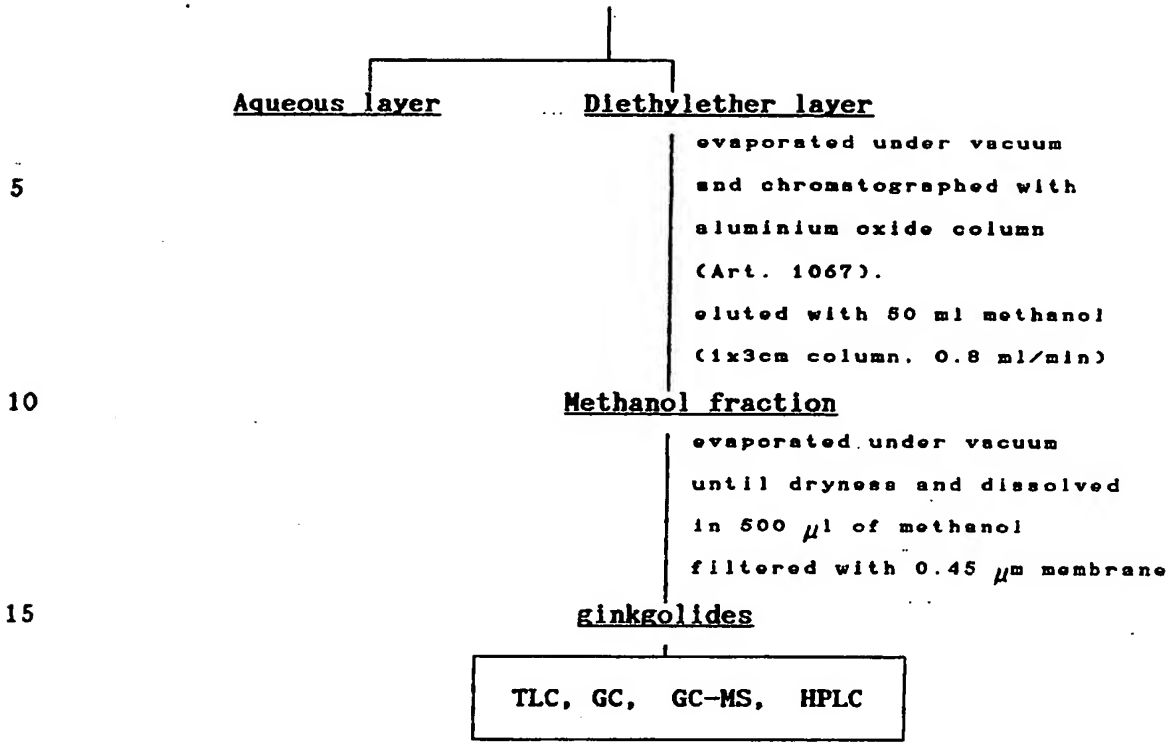
during which cell mass increases 5-6 times.

The chemotherapeutically active ginkgolides produced by the culture are essentially the same in terms of structure as those found in the intact plant.

The recovery of the ginkgolides from the callus or suspension cultured cells may be performed by any conventional procedures as known in the art. For instance, the callus or suspension cultured cells are lyophilized and extracted as shown in Scheme I. The resultant residue containing the ginkgolides was dissolved in methanol, trimethylsilylated and employed as a sample for the GC analysis described hereinafter.

The following detailed examples are intended only to further illustrate the invention and are not intended to limit the scope of the invention which is defined by the claims.





Scheme I. Extraction and purification of ginkgolides from lyophilized callus or suspension cultured cells derived from *Ginkgo biloba*

EXAMPLE 1

Plant material of *Ginkgo biloba* was collected either from the field- grown trees or the seedlings which were obtained either by the germination of *Ginkgo* seeds in soil-less mix consisting of the vermiculite and sand or by the aseptic embryo culture. The plant material consisted of leaves, stem and roots.

The roots were obtained only from the aseptic *Ginkgo biloba* seedlings which were obtained from the embryo culture. The plant materials except the roots from the aseptic embryo culture were surface-sterilized by immersing them into 70% ethanol for 10 sec followed by a 0.25% sodium hypochlorite solution for 8 min, respectively. Then, they were rinsed 4 times with sterilized distilled water. All procedures utilized sterile techniques. The leaves were then cut with a scalpel into approximately 0.7 x

0.7 cm squares and the roots were cut into approximately 0.5 cm in length. The excised explants were transferred by forceps to MS basal medium supplemented with 3% sucrose, 0.8% agar, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxin-HCl, 0.1 mg/L thiamine-HCl, 100 mg/L myo-inositol, 2 mg/L glycine, 1.0 ppm 1-naphthalenacetic acid (NAA) and 0.1 ppm kinetin to induce callus, and cultured at 25 ± 1 ℃ under the dark. Callus proliferation occurred 2 to 3 weeks later. The induction rate of callus at the 4 weeks after the inoculation was about 98%.

The induced callus was maintained at 25 ± 1℃ either under the dark or the light, and was subcultured every three weeks onto fresh MS supplemented medium. They were periodically examined for growth characteristics and the contents of ginkgolides.

EXAMPLE 2

Example 1 was repeated except that the types and concentrations of the auxin used in the MS supplemented medium to find out the effect of types and concentrations of auxin on the induction of the callus. In terms of the types of auxin employed, NAA was the most effective over a concentration ranges from 0.1 to 4.0 ppm (Table III and Fig. 1).

Table III. Effect of types and concentrations of auxin on the induction of callus from the leaves of *Ginkgo biloba*

auxins	concentration (ppm)	induction rate of callus (%)	fresh weight of callus (mg)
IAA	0.1	11.1	-
	0.5	13.0	-
	1.0	33.0	-

	2.0	40.0	-
	4.0	77.3	126 ± 69.5
IBA	0.1	18.2	-
	0.5	81.0	171 ± 102.8
	1.0	87.0	199 ± 64.6
	2.0	90.0	172 ± 48.5
	4.0	90.5	197 ± 41.3
2,4-D	0.1	33.3	-
	0.5	78.3	114 ± 4.9
	1.0	87.0	204 ± 46.7
	2.0	86.4	186 ± 3.0
	4.0	75.0	138 ± 96.9
NAA	0.1	82.6	195 ± 60.2
	0.5	92.0	283 ± 70.4
	1.0	95.0	291 ± 65.4
	2.0	90.5	248 ± 51.5
	4.0	90.9	237 ± 106.9

IAA : Indol-3-ylacetic acid IBA : γ-(Indol-3-yl)butyric acid

2,4-D : 2,4-Dichlorophenoxyacetic acid

NAA : 1-naphthalenacetic acid

Although NAA itself could induce the callus as much as 95% from the leaves of *Ginkgo biloba* at the concentration of 1.0 ppm, the effect of kinetin was examind in various combinations with NAA. The combination of 0.1 ppm of kinetin with 1.0 ppm of NAA provided the optimal condition for the callus induction (Table IV).

Table IV. Effect of various combinations of NAA and kinetin on the extent of the induction of callus from the leaves of *Ginkgo biloba* under the light or dark

	light			dark		
NAA (ppm) / kinetin(ppm)	0.0	1.0	2.0	0.0	1.0	2.0
0.0	-	++	++	-	++	+++
0.1	-	++++	++++	-	++++	++++
0.5	-	++	+++	+	++	+++

- : poor, + : fair, ++ : good, +++ : very good

The concentrations of growth regulators for optimal callus induction were 1.0 to 2.0 ppm for NAA and 0.1 ppm for kinetin.

To determine the effect of different types and concentrations of cytokinin in combination with NAA on the growth of the callus derived from the leaves of *G. biloba*, 50 mg of callus induced in Example 1 was inoculated on fresh MS solid medium supplemented with NAA and kinetin or 6-benzylaminopurine (BA), respectively and cultured for 4 weeks in various concentrations either under the dark or light (Table V-I, -II, Fig. 2).

Table V-I. Effect of different concentrations of NAA and cytokinin on the growth of callus derived from the leaves of *Ginkgo biloba* under the light

auxin (NAA, ppm)	cytokinin (BA, ppm)	fresh weight of callus (mg)
1	0.1	270 ± 58.9
1	0.5	260 ± 30.2
1	1.0	278 ± 27.7

2	0.1	302 ± 88.1
2	0.5	283 ± 104.9
2	1.0	310 ± 89.3
4	0.1	278 ± 59.1
4	0.5	224 ± 98.4
4	1.0	260 ± 61.8

auxin (NAA, ppm)	cytokinin (kinetin, ppm)	fresh wt of callus (mg)
1	0.1	298 ± 102.5
1	0.5	295 ± 75.7
1	1.0	261 ± 77.1
2	0.1	328 ± 82.8
2	0.5	309 ± 71.2
2	1.0	270 ± 65.2
4	0.1	385 ± 43.5
4	0.5	269 ± 45.8
4	1.0	300 ± 75.7

Table V-II. Effect of different concentrations of NAA and cytokinin on the growth of callus derived from the leaves of *Ginkgo biloba* under the dark

auxin (NAA, ppm)	cytokinin (BA, ppm)	fresh weight of callus (mg)
1	0.1	259 ± 37.4
1	0.5	288 ± 68.0

1	1.0	290 ± 77.4
2	0.1	289 ± 70.7
2	0.5	311 ± 74.0
2	1.0	270 ± 39.2
4	0.1	290 ± 38.4
4	0.5	236 ± 23.5
4	1.0	226 ± 43.5

auxin (NAA, ppm)	cytokinin (kinetin, ppm)	fresh wt of callus (mg)
1	0.1	265 ± 66.6
1	0.5	228 ± 48.3
1	1.0	272 ± 55.5
2	0.1	256 ± 55.6
2	0.5	275 ± 58.6
2	1.0	261 ± 76.3
4	0.1	331 ± 59.0
4	0.5	254 ± 44.0
4	1.0	183 ± 36.6

The growth of the callus seemed to be more stimulated with the combination of NAA and kinetin than NAA and BA at all concentration ranges studied. The growth of callus was stimulated by growth regulators with illumination in all the concentration ranges studied (Fig. 2). However, no significant difference could be obtained for the fresh weight of callus after 4 weeks of cultural period (Table V-I,-II) This seems to indicate that growth is regulated by the types and concentrations of auxin rather

than the cytokinin used.

EXAMPLE 3

Example 1 was repeated except that MS salt mixture was substituted for that of Anderson's (And), Chu(N_6), Eriksson (ER), Gamborg's B-5 (B-5), Heller's (H), Schenk and Hildebrandt (SH) and White's (W), respectively to find out the effect of different types of culture media on the induction of callus from the leaves of *Ginkgo biloba*. Among the 8 different media used, the induction rate of callus on And, ER and SH at the 4 weeks after the inoculation was almost the same as that on MS. However, callus was rarely induced on Heller's or White's medium. It is of interest to note that adventituous buds began to form within 3 weeks following the first inoculation on SH medium(Table VI, Fig. 3).

Table VI. Effect of the different types of culture media on the induction of callus derived from the leaves of *Ginkgo biloba*

Replication	Media							
	MS	B-5	ER	W	And	N_6	H	SH
1	++	++	++	-	++	++	+	++
2	++	++	+++	+	+++	++	-	++
3	+++	+	++	-	++	+	-	++

- : poor, + : fair, ++ : good, +++ : very good

EXAMPLE 4

Subcultures from the callus of Example 1 were divided into 8 different solid media with the same supplementation as in Example 1. Growth of callus on MS medium was found to be the most rapid. Growth of the callus was fairly good on medium of And, B-5, N_6 or SH. However, very slow growth of the callus on ER, Nitsch and Nitsch (N-N) or White's medium was observed (Table VII, Fig. 4).

Table VII. Effect of the different types of culture media on the growth of callus derived from the leaves of *Ginkgo biloba*

Replication	Media							
	MS	B-5	ER	W	And	N_6	N-N	SH
1	+++	++	++	++	+++	+++	+	+++
2	+++	+++	+	+	++	++	+	++
3	+++	++	+	-	+++	+++	+	+++

- : poor, + : fair, ++ : good, +++ : very good

The growth rate was determined with the 6th subcultured callus of Example 1 by measuring the fresh weight of callus over a total cultural period of 28 days with 7 days interval (Table VIII).

Table VIII. The fresh weight of callus derived from the leaves of *Ginkgo biloba*

Cultural period (day)	Fresh weight of callus (mg)
0	570.6 ± 99.3
7	883.2 ± 125.1

14	1226.8 ± 159.1
21	1647.9 ± 152.3
28	1975.1 ± 337.8

EXAMPLE 5

Suspension cultures were easily initiated with 10th subcultured callus from Example 1. Suspension cultures were performed in 40 ml of medium for Example 1 except agar in 125-ml Erlenmeyer flask or 80 ml of the medium in 250-ml Erlenmeyer flask with agitation at the rate of 100-110 rpm on a reciprocal shaker at 25 ± 1°C under the illumination. The culture was subsequently subcultured at 2-week interval. The fresh weight of the suspension cultured cells was measured every 3 days to obtain the growth curve of suspension cultured cells. A typical graph of the growth of *Ginkgo biloba* cells derived from the leaves in suspension culture is shown in Fig. 5. The cultured cells have reached the stationary phase at the 12th day of the culture.

EXAMPLE 6

The aseptic plantlet of *Ginkgo biloba* was obtained by the embryo culture on MS solid medium without the supplementation of growth regulators. The effect of activated charcoal on the initiation and development of the root obtained from the embryo culture of *Ginkgo biloba* was investegated. The development of the roots in *Ginkgo biloba* plantlet from the embryo culture was much efficient by the addition of 0.3% activated charcoal into the MS solid medium (Fig. 6). The amounts of ginkgolides A (GKA), B (GKB) and C (GKC) in the roots and leaves of 6-week-old-plantlets obtained by the embryo culture under the dark or illumination were as follows:

Illumination	Organ	Amounts of ginkgolides (10^{-2}% of dry weight)		
		GKA	GKB	GKC
dark	roots	10.3 ± 1.98	14.5 ± 4.27	1.2 ± 0.58
light		10.5 ± 0.14	3.4 ± 0.99	2.1 ± 0.23
dark	leaves	2.3 ± 0.35	2.2 ± 0.26	0.2 ± 0.09
light		10.4 ± 2.44	14.0 ± 2.51	10.3 ± 0.98

The results showed that the roots and leaves of the plantlet obtained by the embryo culture contained almost the same level of ginkgolides in the roots and leaves of field-grown Ginkgo tree.

EXAMPLE 7

The roots from Example 6 were used to initiate the formation of callus by the procedures of Example 1 except surface sterilization. The induction rate of callus from the roots of plantlet was about 60% in the period of 4 weeks under the dark (Fig. 7). The callus was placed into suspension cell culture. The amounts of ginkgolide B in the 4th subcultured suspension cells were 3.83 x 10^{-3}% of dry weight.

EXAMPLE 8

The ginkgolides production by callus or suspension cultured cells was identified by GC-MS with authentic ginkgolides A, B and C. The callus or suspension cultured cells were lyophilized and then pulverized. The resultant powder was extracted with aceton-water mxture (1 : 1, v/v) with sonication at an ambient

temperature as described in Scheme I. The supernatant was evaporated under vacuum until the complete removal of aceton. The remaining aqueous layer was extracted with n-hexane to remove the non-polar substances. The partially purified remaining aqueous layer was adjusted to pH 2.0 with 1N HCl and extracted again with ethylacetate. The ethylacetate layer was evaporated to dryness under vacuum. The residue was suspended in deionized water and extracted with diethylether. The ether extract was evaporated to dryness under vacuum. The residue was redissolved in methanol and passed through a alumina column with methanol as an eluent. The eluent was evaporated to dryness under vacuum and the residue was redissolved in methanol and then filtered through a 0.45 μM membrane.

The identification of ginkgolides A, B and C from *Ginkgo* tissue cultures was accomplished by GC-MS with authentic ginkgolides A, B and C. For the analysis with GC-MS, the obtained ginkgolides fraction as above mentioned was reacted with silylating agent (Tri-Sil BSA DMF, Pierce Chemicals, Rockford, IL, USA) at 73°C for 1 hr. GC-MS analysis of the extracts were performed with a Hewlett-Packard model HP 5985 series II GC directly interfaced to a VG Trio II mass spectrometer. The GC-MS conditions are as follows: SE-54 capillary column (0.2 mm x 17 m); split ratio of 1 : 10 ; interphase temperature of 300°C; ion source temperature of 300°C and detector temperature of 300°C. The column temperature was increased 20°C/min from 100°C to 300°C. Helium was used as the carrier gas at a flow rate of 0.89 ml/min.

Using the above conditions, the authentic ginkgolides A, B and C have retention times of 11.6 min, 12.1 min and 12.2 min, respectively (Figs. 8-1A,-1B and -1C). As shown in Figs. 8-2A, -2B, -2C, -3A and -3B, GC-MS spectra strongly indicates the formation of these ginkgolides in the cultured cells as well as the callus.

EXAMPLE 9

The amounts of the ginkgolides recovered from callus or suspension cultured cells were determined by gas chromatography. The sample preparation of ginkgolides fraction from the callus or suspension cultured cells for the GC analysis was the same as in Example 8. The identification and quantitation of ginkgolides A and B were accomplished by GC on a Hewlett-Packard model HP 5985 series II equipped with a flame ionization detector (FID) and OV-1 capillary column (0.2 mm x 30 m). The GC conditions are as follows: injection temperature of 295°C; column temperature of 280°C; detector temperature of 295°C and at a flow rate of 0.5 ml/min using N_2 as a carrier gas.

Using the above conditions, the authentic ginkgolides A and B have retention times of 18.2 min and 20.7 min, respectively. (Fig. 9-1). The amounts of ginkgolides A and B are calculated from the measurement of the area of the corresponding peaks in the chromatogram. The gas chromatogram of the extract of suspension cultured cells which were derived from the roots exhibits that the cultured cells contained components which had identical retention times of 18.2 and 20.7 min for the authentic ginkgolides A and B, respectively (Fig. 9-2). To support this result, co-injection of authentic ginkgolide B with the extract of the suspension cultured cells which were derived from the roots was performed (Fig. 9-3). The peak hight of the component suspected to be ginkgolide B in the extract of the suspension cultured cells which were derived from the roots increased by the co-injection of the authentic ginkgolide B and the peak was apparently symmetrical. The gas chromatogram of the extract of the leaf derived from suspension cultured cells shows that the cultured cells contained components which had identical retention times of 18.2, 20.7 and 21.7 min for the authentic ginkgolides A, B and C, respectively (Fig. 9-4). The amounts of respective ginkgolides A and B in the callus or suspension cultured cells were in the range of 0.99 to 3.83 x 10^{-3}% of dry weight.

EXAMPLE 10

Ginkgo leaves and roots from Examples 1 and 6 were used to initiate the formation of callus by the procedures of Examples 1 and 7. Suspension cultures were initiated with the callus from Example 1 and 7 as in Example 5. Analysis of the culture supernatant by GC was positive for ginkgolides A and B. (Figs. 9-5, -6).

EXAMPLE 11

The identification of ginkgolides recovered from callus or suspension cultured cells was performed with high performance liquid chromatography. The sample preparation for ginkgolides fraction from the callus or suspension cultured cells for the HPLC analysis was the same as in Example 8. The identification of ginkgolides A and B was accomplished by HPLC on a Shimadzu pump equipped with a Shimadzu SPD-6A UV detector and a C_{18} reversed-phase column (5 x 250 mm, 10 um). An isocratic flow of the mixture of isopropanol : water (1 : 9 , v/v) was used to optimize the seperation of each ginkgolide from the ginkgolides fraction. The retention times of the authentic ginkgolides A and B were 26.0 min and 28.5 min, respectively (Fig.10-1). Dection at 220 nm was found to be the optimal wavelength.

The HPLC chromatogram of the extract of the callus from Example 1 also indicate the formation of ginkgolides A and B (Fig. 10-2).

EXAMPLE 12

The 5th subcultured callus from Example 7 was introduced to suspension culture and subcultured 8 times. Example 4 was repeated with the 8th subcultured suspension cells in 80 ml of the medium contained in 250-ml Erlenmeyer flask except that the concentration

of sucrose was varied in the ranges from 20 g/l to 60 g/l. The entire experiment was performed in triplicate. The dry weight values (mg cells/flask) and ginkgolides contents at the 14 days of the culture were as follows:

Sucrose (g/l)	Dry weight (mg)	GKA		GKB	
		10^{-4}% of dry weight	μg/flask	10^{-4}% of dry weight	μg/flask
20	427 ± 73.2	3.4 ± 0.17	1.46	2.3 ± 0.19	0.98
30	670 ± 42.5	4.6 ± 0.41	3.04	5.4 ± 0.40	3.62
40	666 ± 97.5	7.5 ± 0.52	5.01	4.0 ± 0.17	2.65
60	474 ± 19.5	5.8 ± 0.43	2.74	3.8 ± 0.50	1.78

The optimum sucrose concentration in the medium for ginkgolide B production was 30 g/l.

EXAMPLE 13

The 3rd subcultured callus from Example 1 was introduced to suspension culture and subcultured 5 times. Example 5 was repeated with 1.5 g (fresh weight) of the 5th subcultured suspension cells in 80 ml of the medium contained in 250-ml Erlenmeyer flask except that the concentration of NAA was varied in the ranges from 1.0 ppm to 8.0 ppm. The entire experiment was performed in triplicate. The dry weight values (mg cells/flask) and ginkgolides contents at the 14 days of the culture were as follows:

NAA (ppm)	Dry weight (mg)	GKA	GKB

		10^{-4}% of dry weight	μg/flask	10^{-4}% of dry weight	μg/flask
1.0	702 ± 46.4	13.6 ± 3.77	9.54	15.2 ± 1.50	10.65
2.0	627 ±123.3	6.8 ± 0.33	4.26	15.2 ± 0.71	9.50
4.0	673 ±110.0	7.5 ± 2.6	5.06	6.6 ± 0.99	4.45
8.0	358 ± 44.5	trace	trace	4.7 ± 0.37	1.66

The optimum NAA concentration in medium for both ginkgolides A and B production was 1.0 ppm. However, the dry weight values showed the concentrations of NAA in the ranges from 1.0 to 4.0 ppm were favorable to cell growth exhibiting no significant differences.

It is understood that the foregoing detailed description is given merely by way of illustration and that modification and variation may be made therein without departing from the spirit and scope of the invention.

CLAIMS

What is claimed is :

1. A method of producing ginkgolides and unique C_{20} lactone cage-like molecules, incorporating a ter-butyl group and six fused five-membered rings, including three gamma-lactone, a tetrahydrofuran and a spiro-(4,4)-nonane unit containing diterpene compounds from cell cultures of *Ginkgo biloba* comprising the steps of:
   a. Providing living tissue of said *Ginkgo biloba;*
   b. Providing a nutrient culture medium suitable for said callus formation from said tissue and for suspension cells growth;
   c. culturing said tissue on said medium to produce calli or cell suspensions derived therefrom and
   d. recovering said ginkgolides from said calli or suspension cultures

2. The method as described in claim 1 wherein said living tissue in step (a) is selected from the group consisting of leaves, stem tissue and roots of field-grown Ginkgo tree or plantlet obtained from the germination in the laboratory or aseptic embryo culture.

3. The method as described in claim 1 wherein said recovery is from said calli.

4. The method as described in claim 1 wherein said recovery is from said cell suspension.

5. The method as described in claim 1 wherein said recovery is from leaf , stem and root tissues of said Ginkgo plantlet obtained by the aseptic embryo culture.

6. The method as described in claim 1 wherein said cell culture comprises cells held in a matrix formed by a gelling agent.

7. The method as described in claim 6 wherein said matrix is formed from agar.

8. The method as described in claim 1 wherein said ginkgolides are ginkgolides A, B, and C.

9. The method as described in claim 1 wherein an inducer or precursor is added to step (c) in an amount sufficient to increase ginkgolides production.

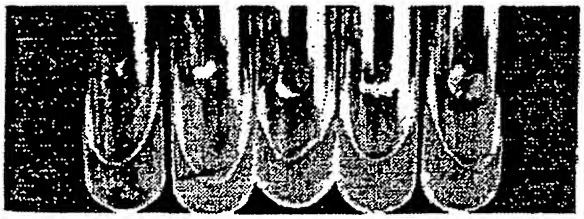
10. The method as described in claim 1 wherein said medium is MS medium, with naphthalene acetic acid and kinetin added as growth regulators.

11. The method as described in claim 1 wherein said medium is SH medium, with naphthalene acetic acid and kinetin added as growth regulators.

12. The method as described in claim 1 wherein said recovery is accomplished by extracting said ginkgolides from calli or suspension cultured cells or culture medium or leaf, stem and root tissues of Ginkgo plantlet obtained by the aseptic embryo culture.

FIG.1

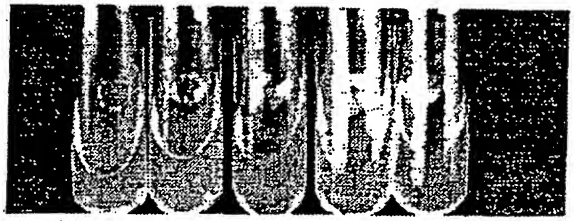
(a)



0.1 0.5 1.0 2.0 4.0 mg/l

IAA

(b)



0.1 0.5 1.0 2.0 4.0 mg/l

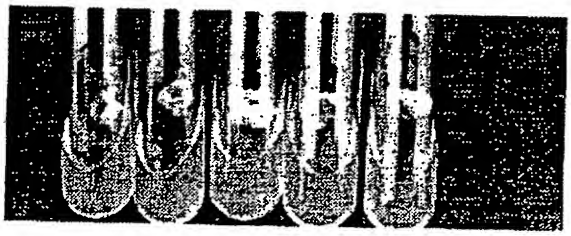
2,4-D

(c)

0.1 0.5 1.0 2.0 4.0 mg/l

IBA

(d)



0.1 0.5 1.0 2.0 4.0 mg/l

NAA

FIG.2

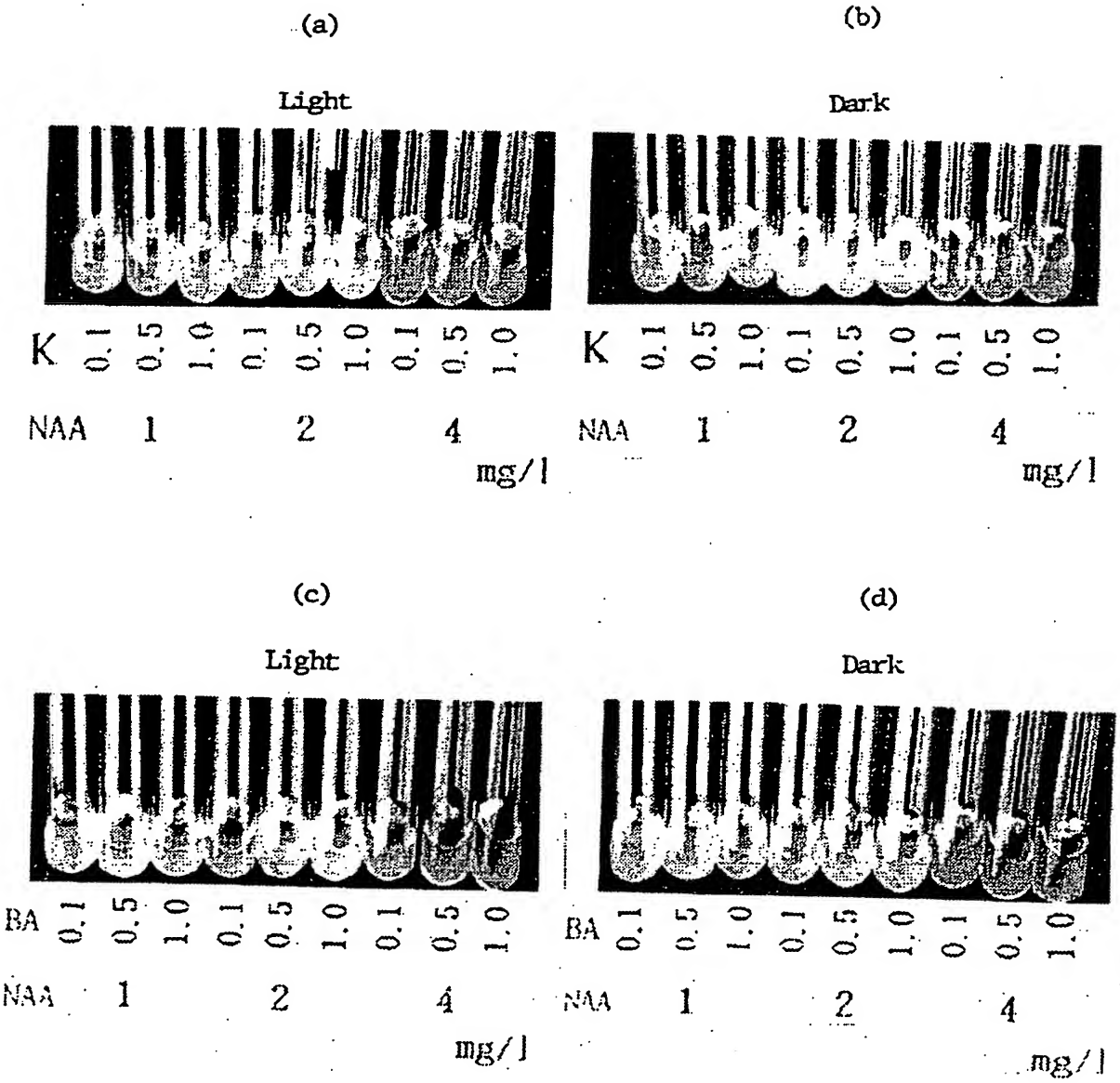

(a)
Light
K 0.1 0.5 1.0 0.1 0.5 1.0 0.1 0.5 1.0
NAA 1 2 4
mg/l
(b)
Dark
K 0.1 0.5 1.0 0.1 0.5 1.0 0.1 0.5 1.0
NAA 1 2 4
mg/l
(c)
Light
BA 0.1 0.5 1.0 0.1 0.5 1.0 0.1 0.5 1.0
NAA 1 2 4
mg/l
(d)
Dark
BA 0.1 0.5 1.0 0.1 0.5 1.0 0.1 0.5 1.0
NAA 1 2 4
mg/l

FIG.3

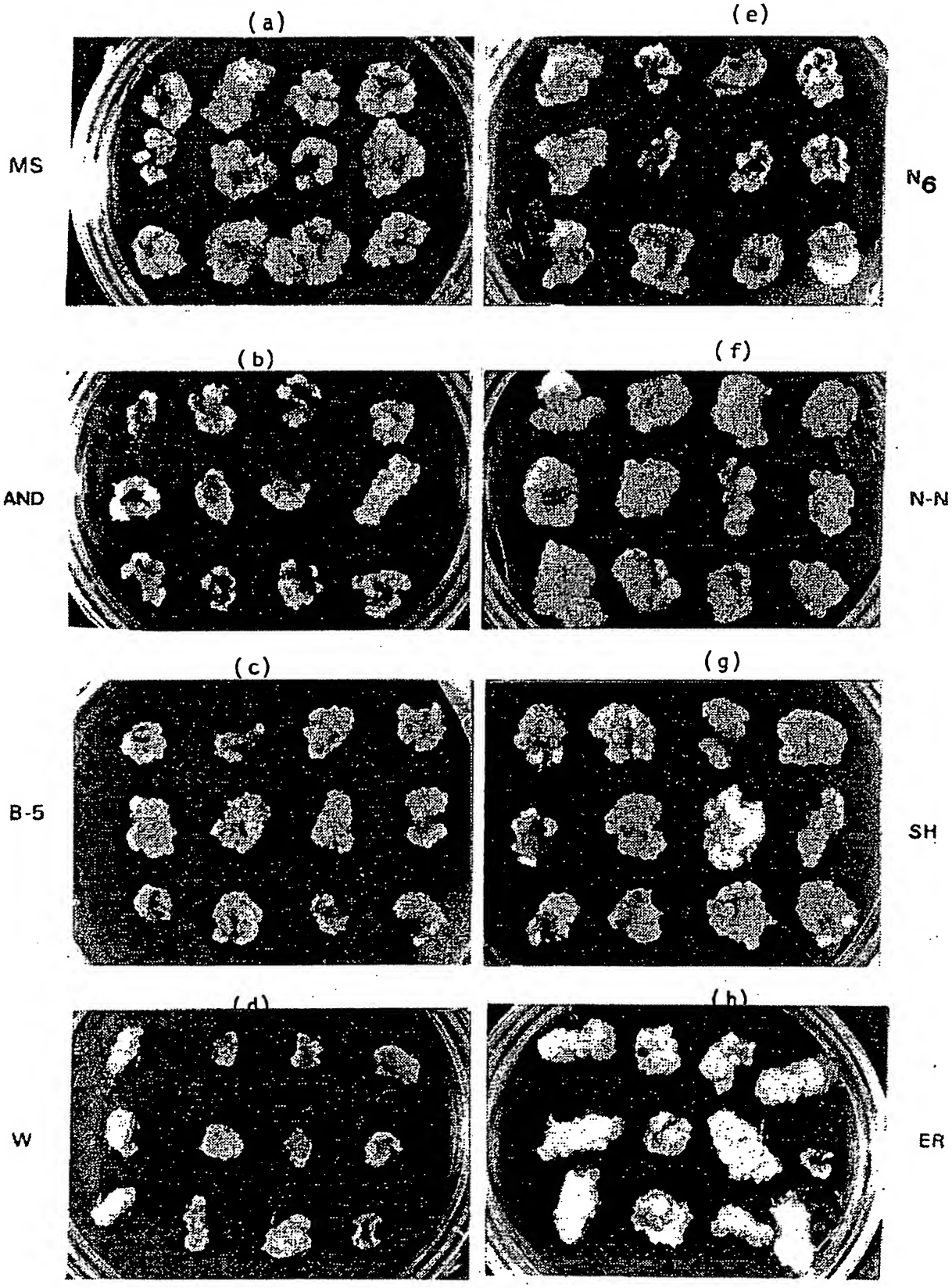
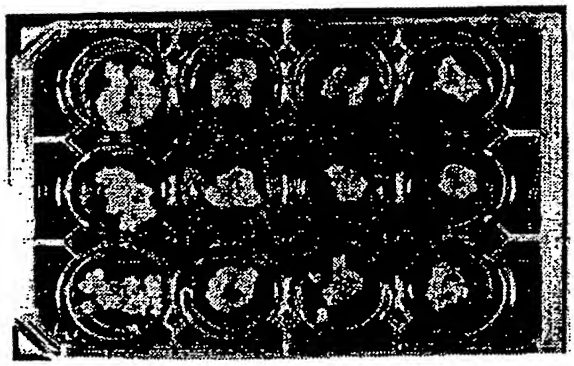


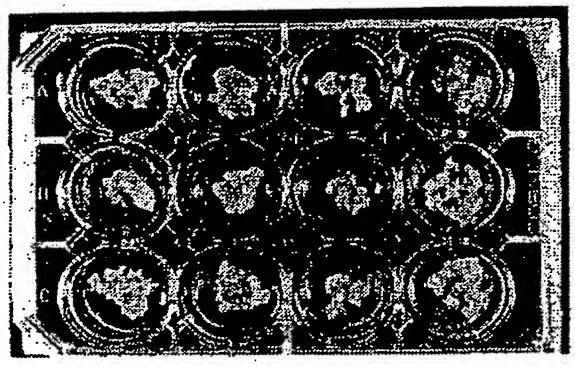
FIG.4

(a)



A B C D

(b)



E F G H

FIG.5

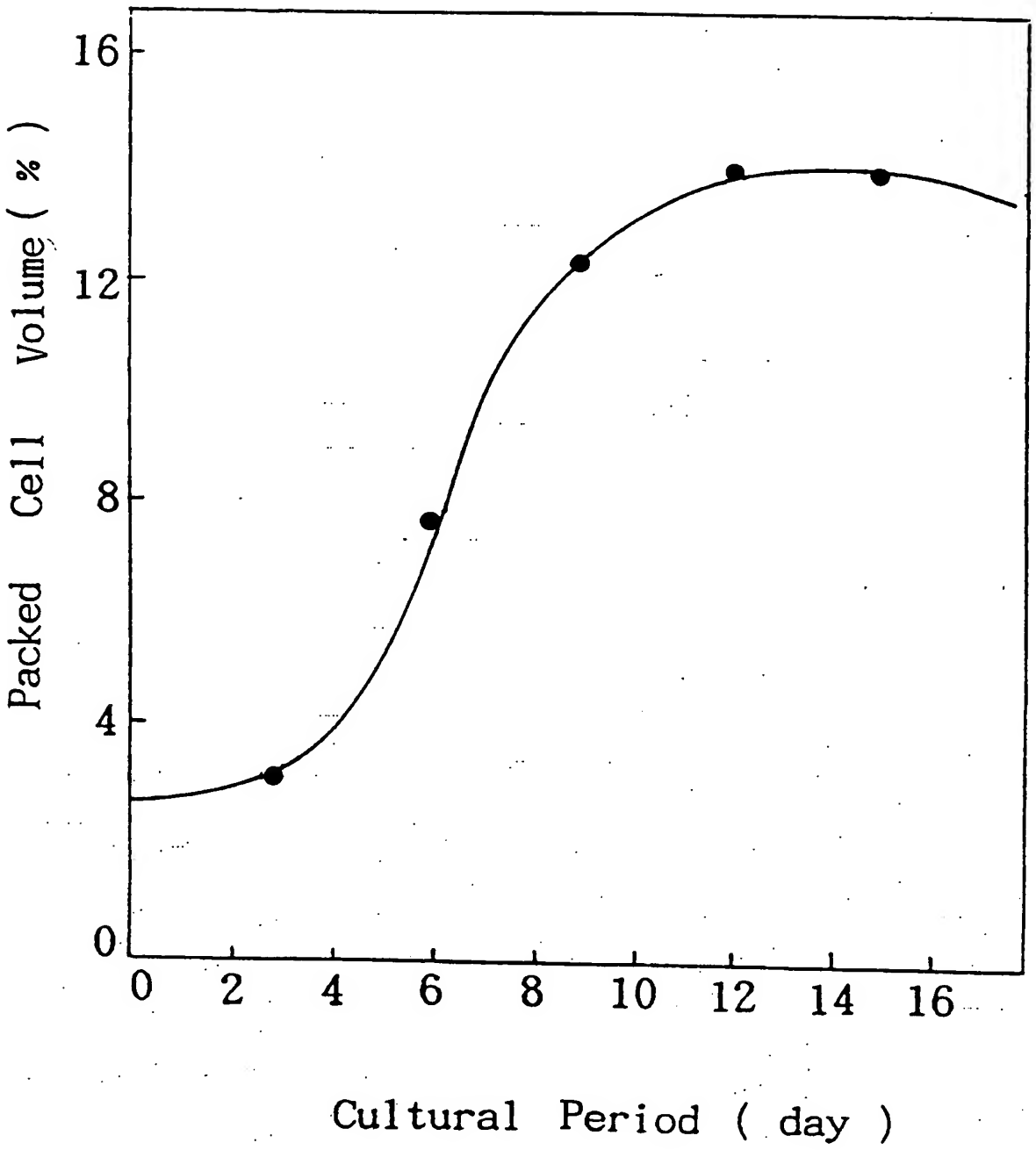
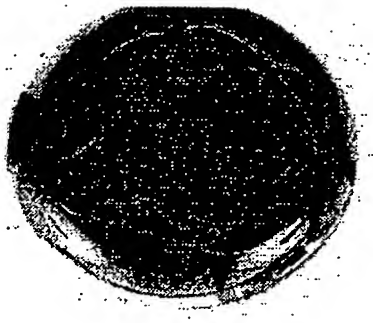

Packed Cell Volume (%)
16
12
8
4
0
0 2 4 6 8 10 12 14 16
Cultural Period (day)

FIG.6



A

B

FIG.7

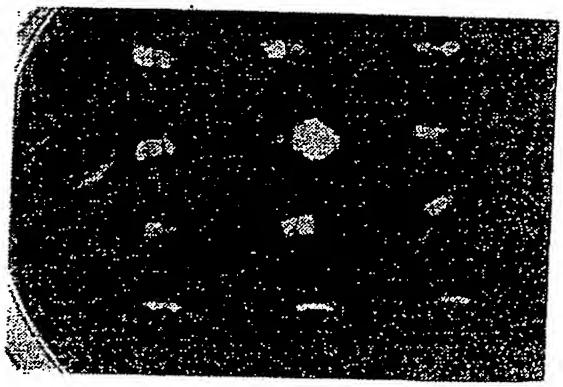


FIG.8-1A

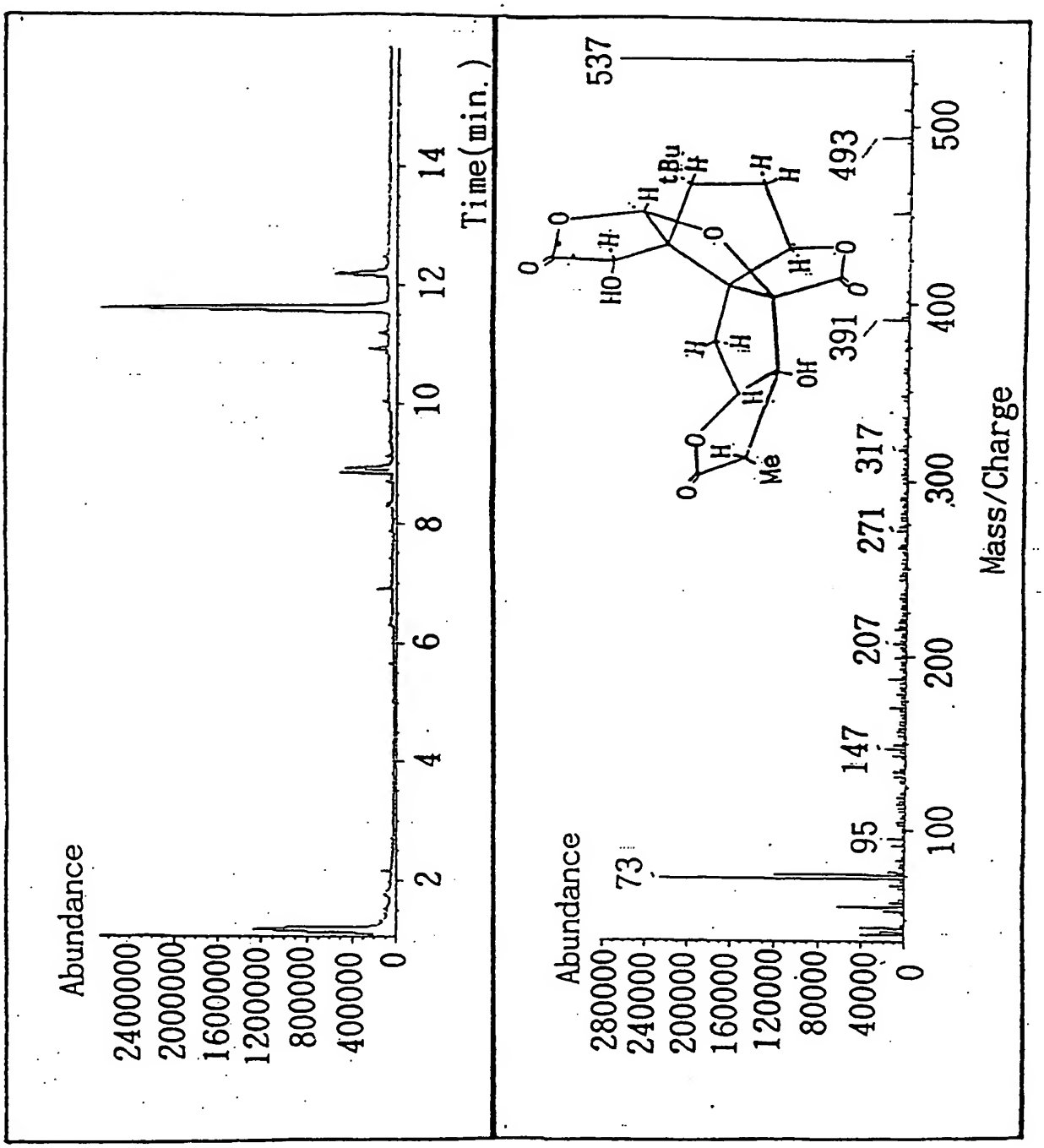

Abundance
2400000
2000000
1600000
1200000
800000
400000
0
2
4
6
8
10
12
14
Time(min.)
Abundance
280000
240000
200000
160000
120000
80000
40000
0
73
95
147
207
271
317
391
493
537
100
200
300
400
500
Mass/Charge
HO
O
tBu
H
OH
Me

FIG.8-1B

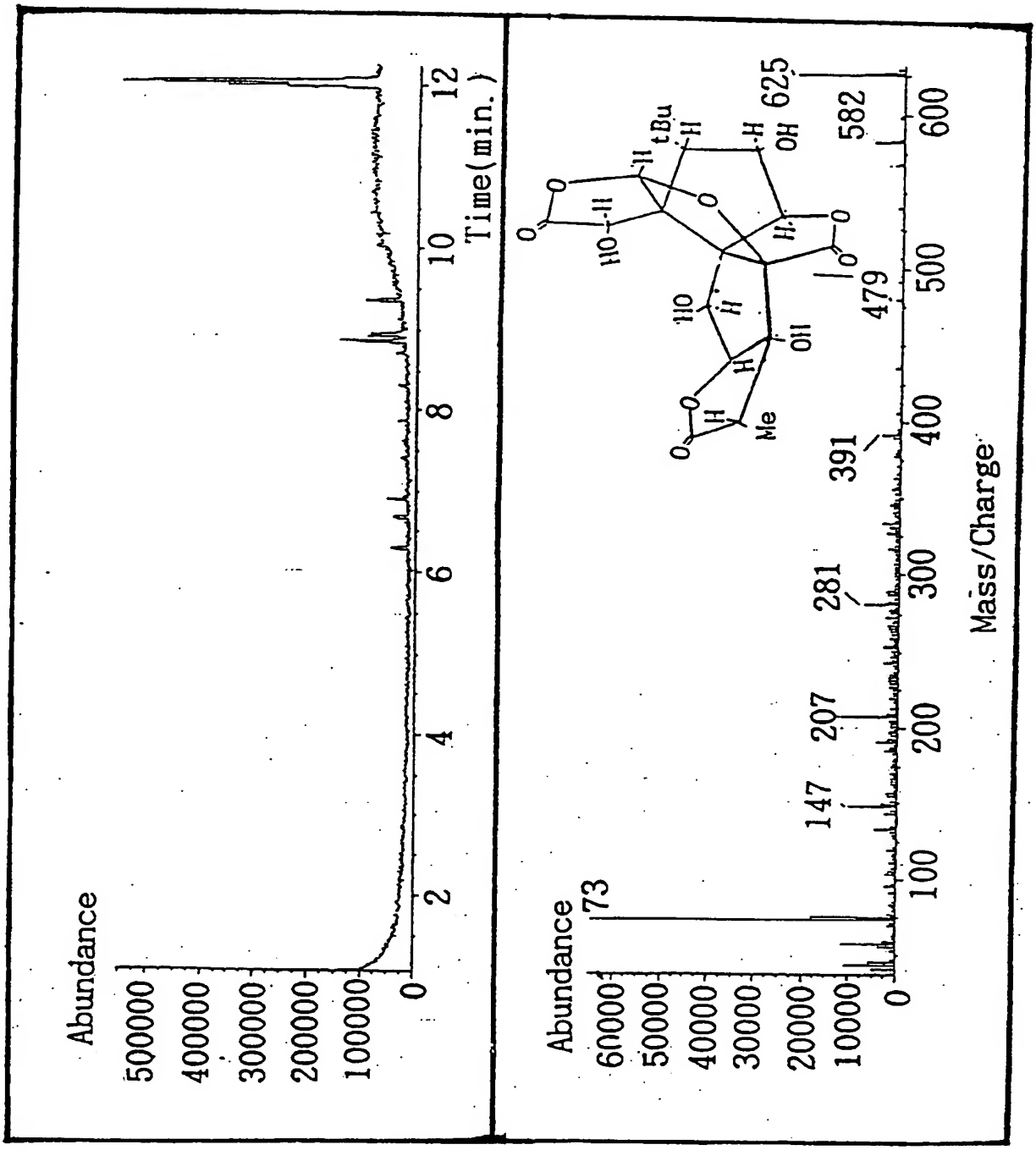

Abundance
500000
400000
300000
200000
100000
0
2
4
6
8
10
12
Time(min.)
Abundance
60000
50000
40000
30000
20000
10000
0
73
147
207
281
391
479
582
625
100
200
300
400
500
600
Mass/Charge
tBu
OH
Me

FIG.8-1C

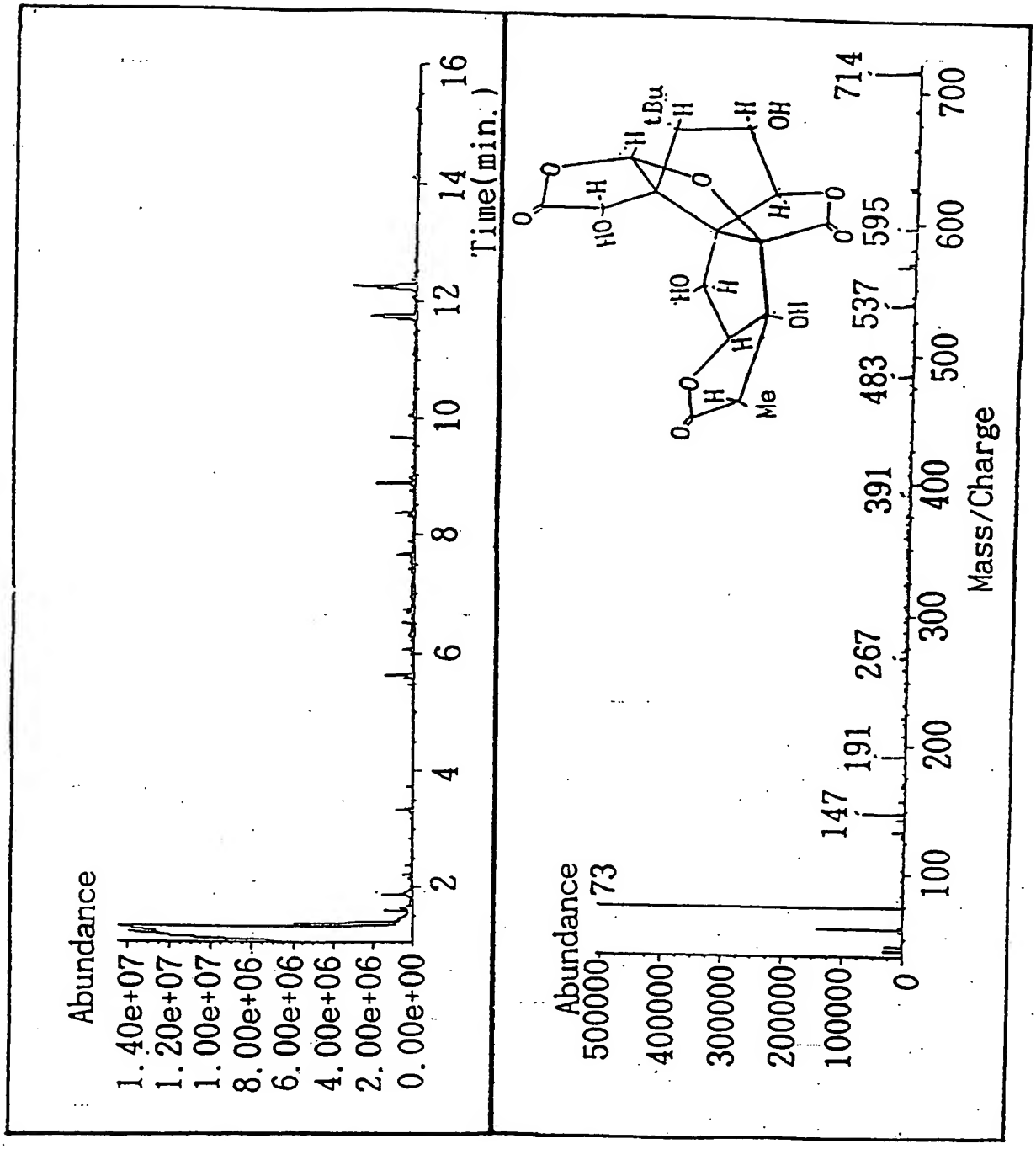

Abundance
1.40e+07
1.20e+07
1.00e+07
8.00e+06
6.00e+06
4.00e+06
2.00e+06
0.00e+00
2
4
6
8
10
12
14
16
Time(min.)
Abundance
500000
400000
300000
200000
100000
0
73
147
191
267
391
483
537
595
714
100
200
300
400
500
600
700
Mass/Charge
t.Bu
OH
HO
Me

FIG.8-2A-a

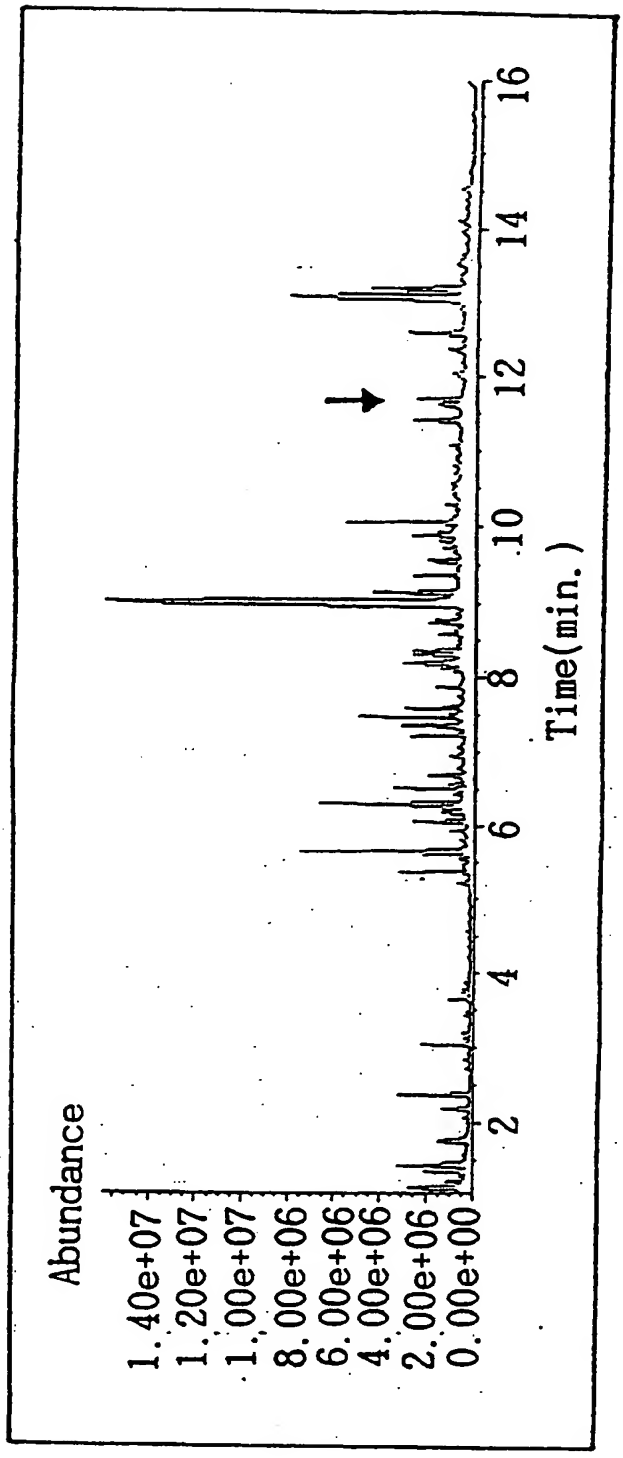

Abundance
1.40e+07
1.20e+07
1.00e+07
8.00e+06
6.00e+06
4.00e+06
2.00e+06
0.00e+00
2
4
6
8
10
12
14
16
Time(min.)

FIG.8-2A-b

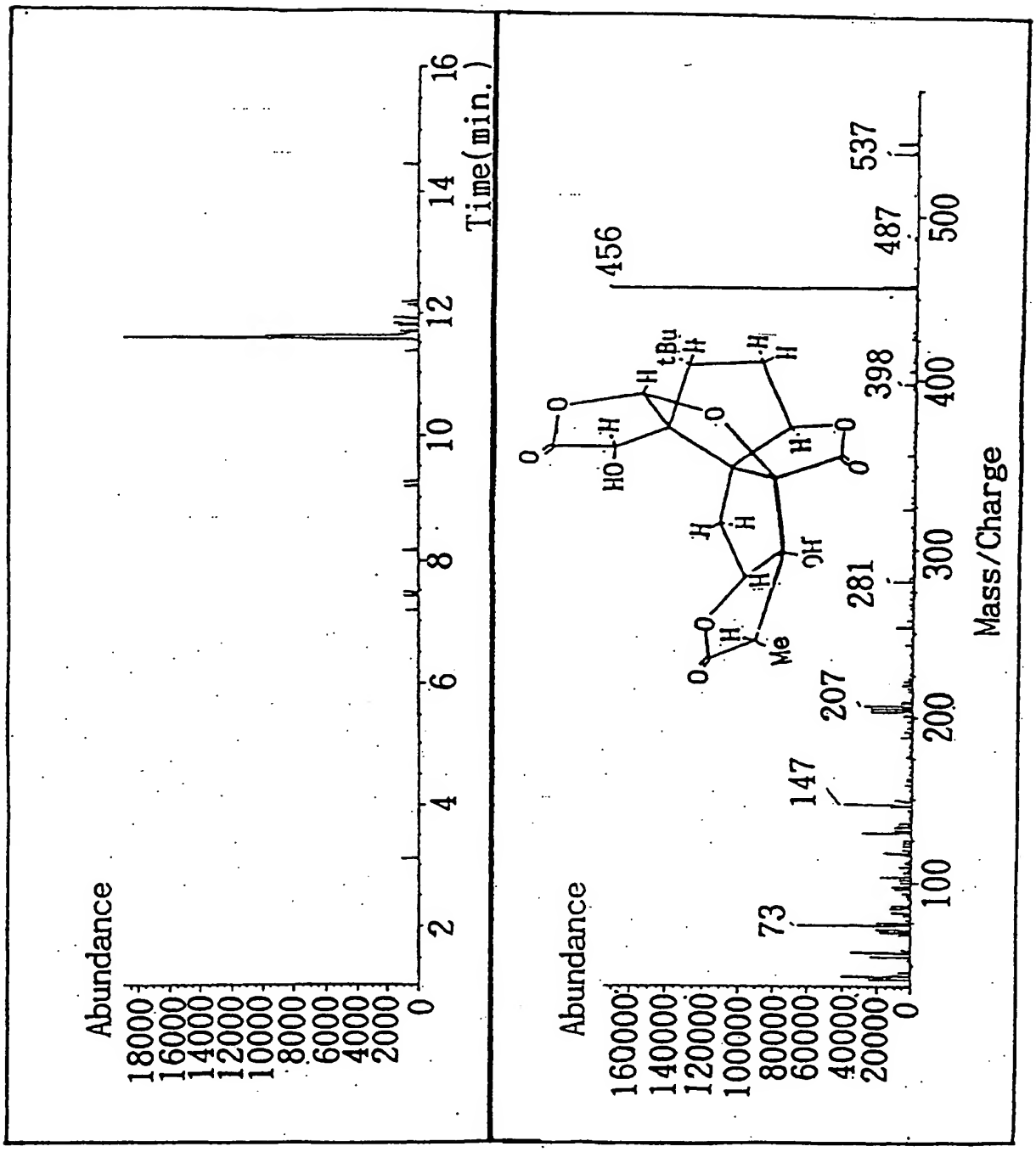

Abundance
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
2
4
6
8
10
12
14
16
Time(min.)
Abundance
160000
140000
120000
100000
80000
60000
40000
20000
0
73
147
207
281
398
456
487
537
100
200
300
400
500
Mass/Charge
HO
tBu
OH
Me

FIG.8-2B-a

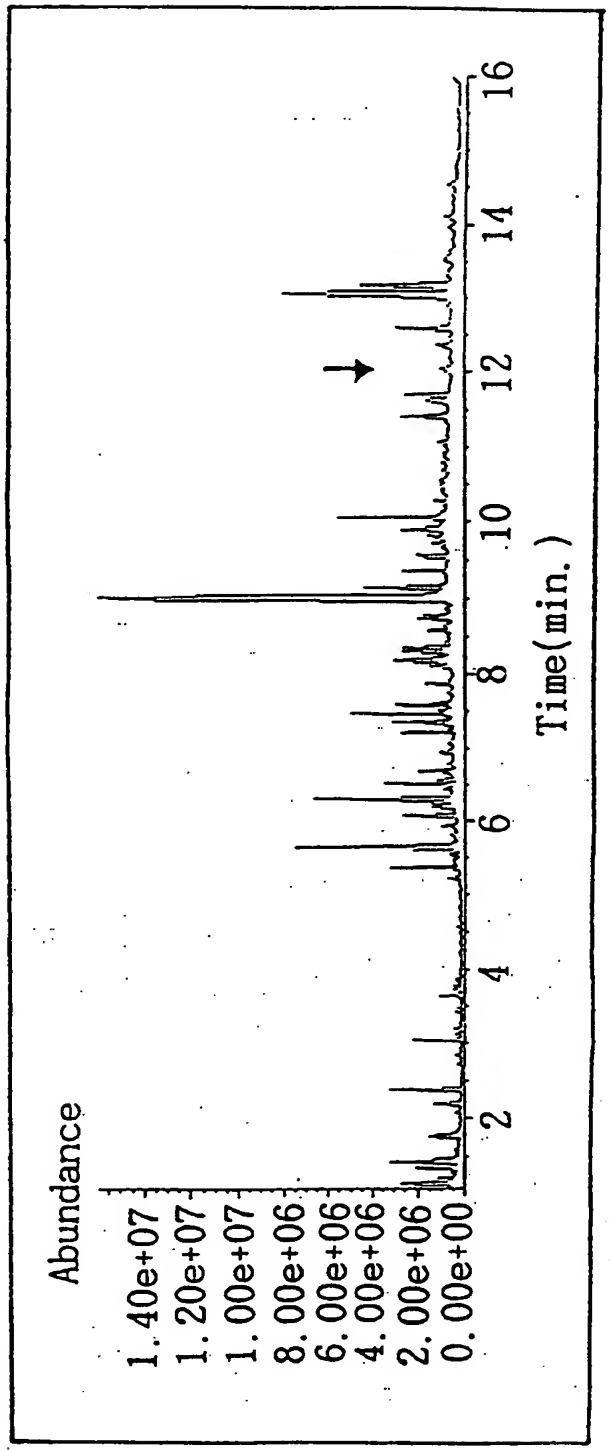

Abundance
1.40e+07
1.20e+07
1.00e+07
8.00e+06
6.00e+06
4.00e+06
2.00e+06
0.00e+00
2
4
6
8
10
12
14
16
Time(min.)

FIG.8-2B-b

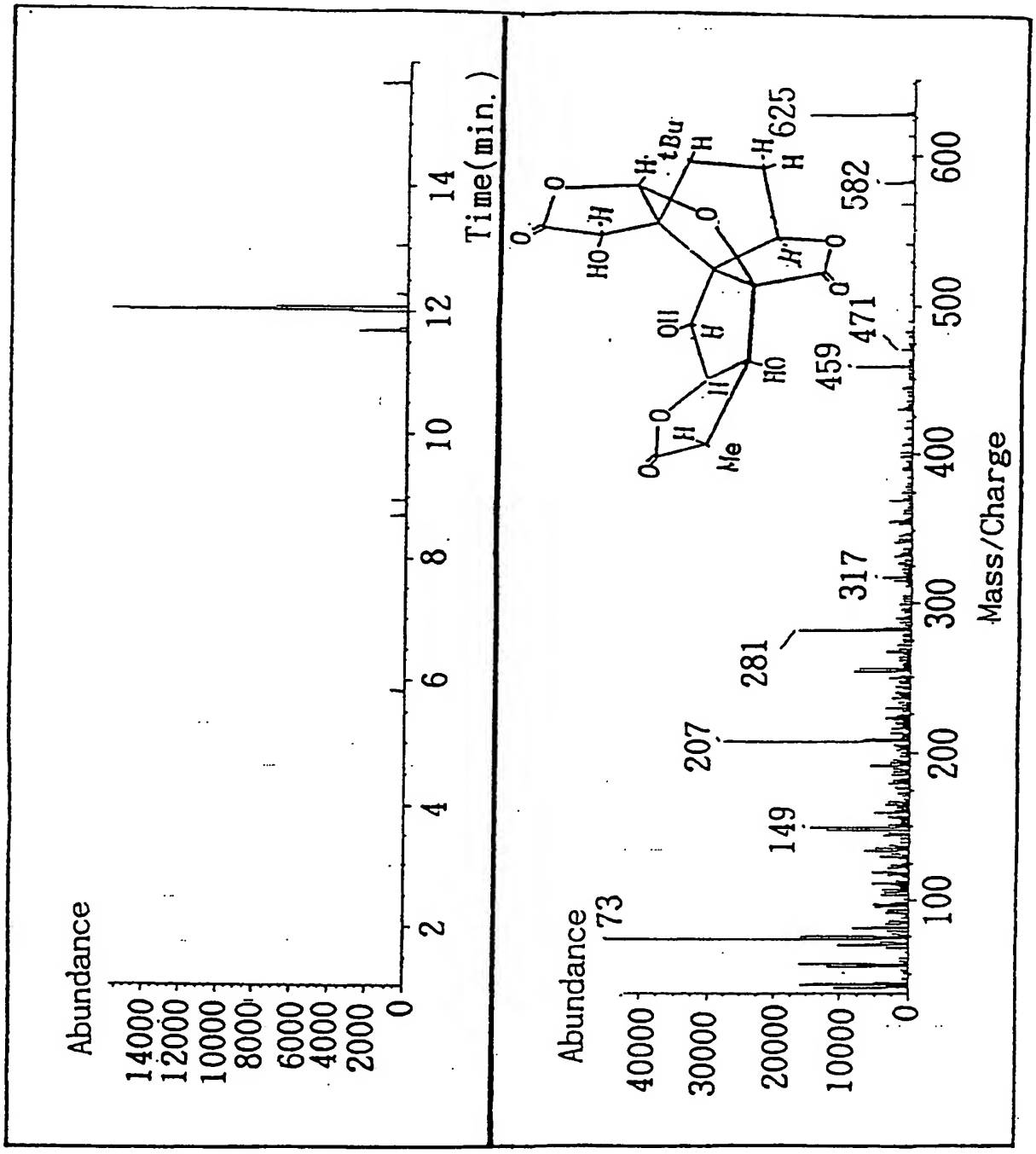


FIG.8-2C-a

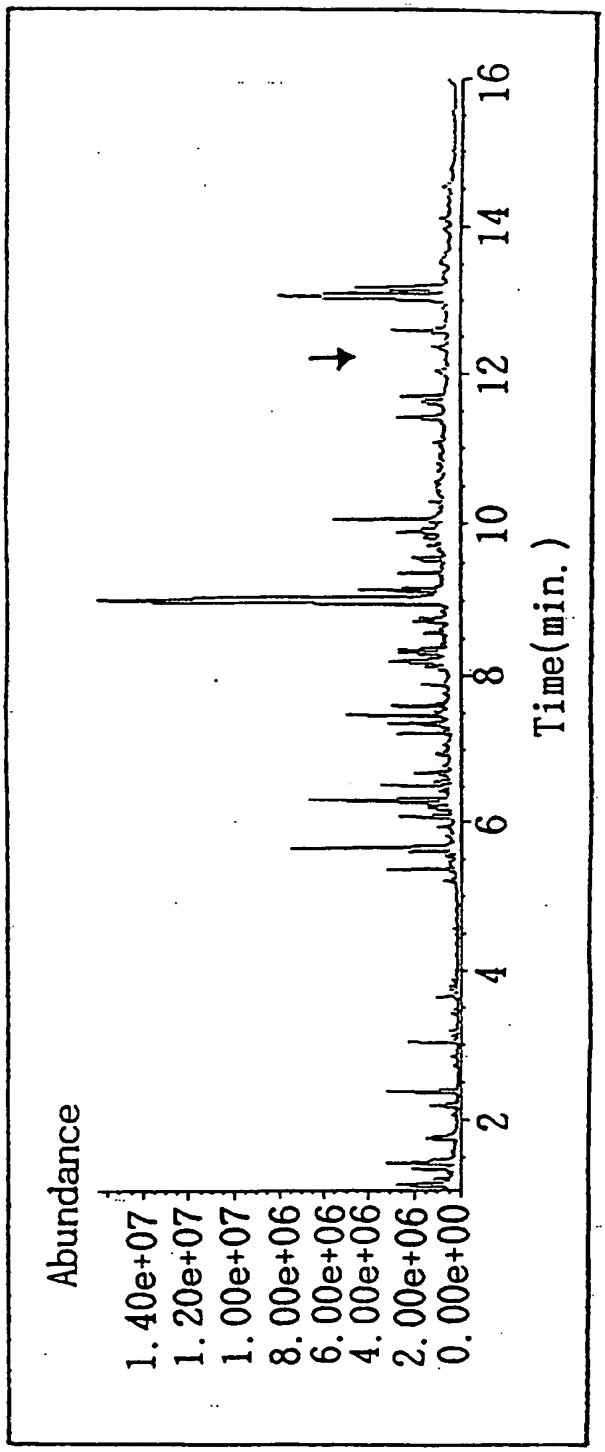
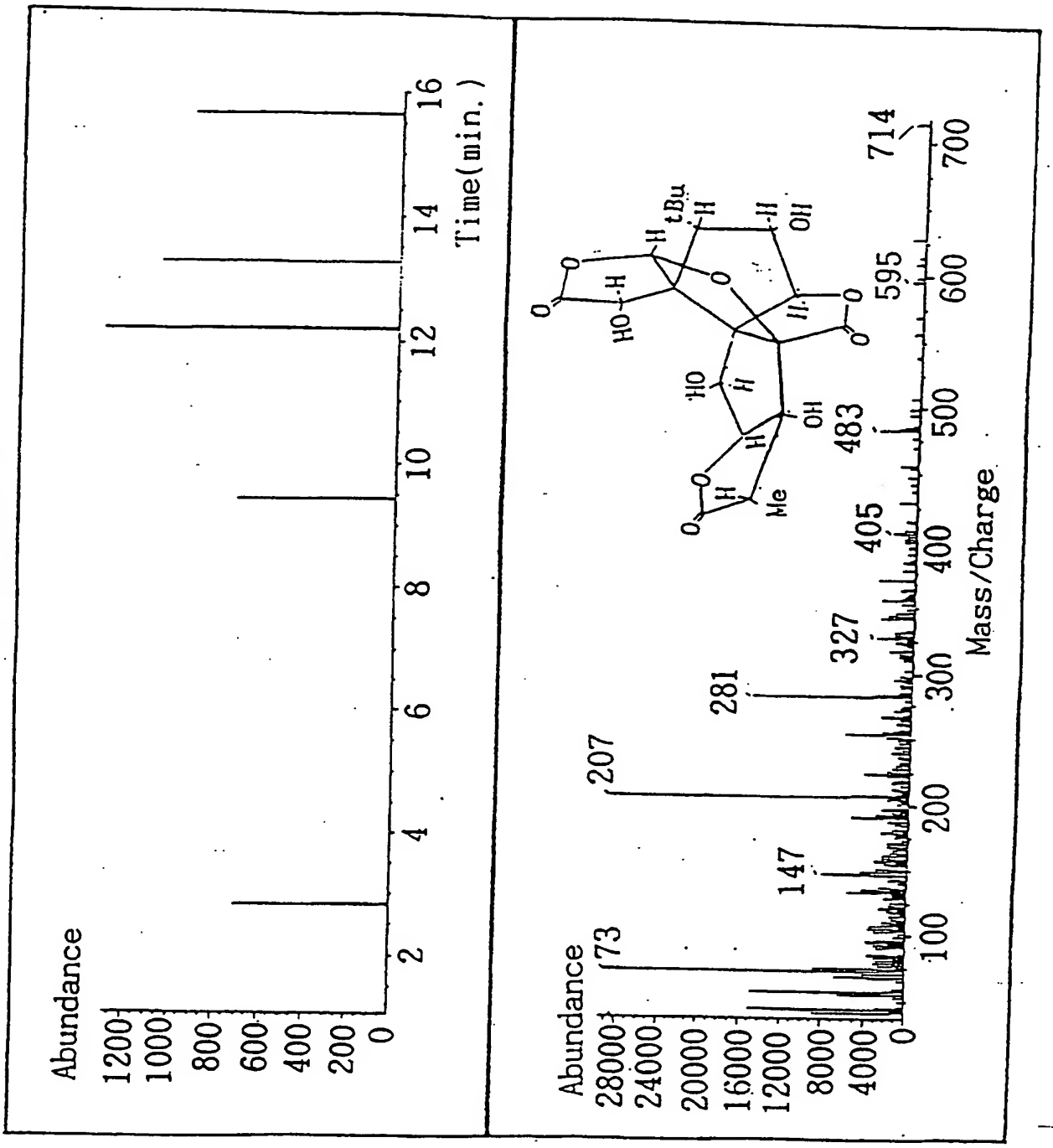

Abundance
1.40e+07
1.20e+07
1.00e+07
8.00e+06
6.00e+06
4.00e+06
2.00e+06
0.00e+00
2
4
6
8
10
12
14
16
Time(min.)

FIG. 8-2C-b



LAST ADDED SHEET

FIG.8-3A-a

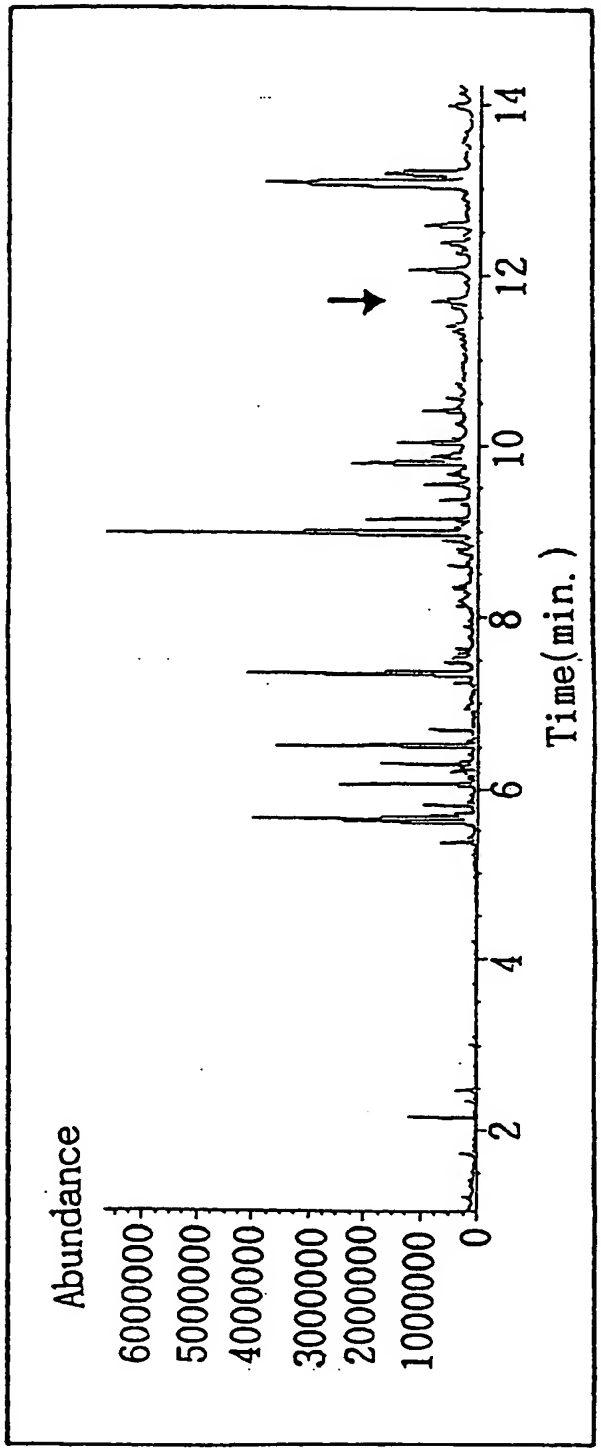

Abundance
6000000
5000000
4000000
3000000
2000000
1000000
0
2
4
6
8
10
12
14
Time(min.)

FIG.8-3A-b

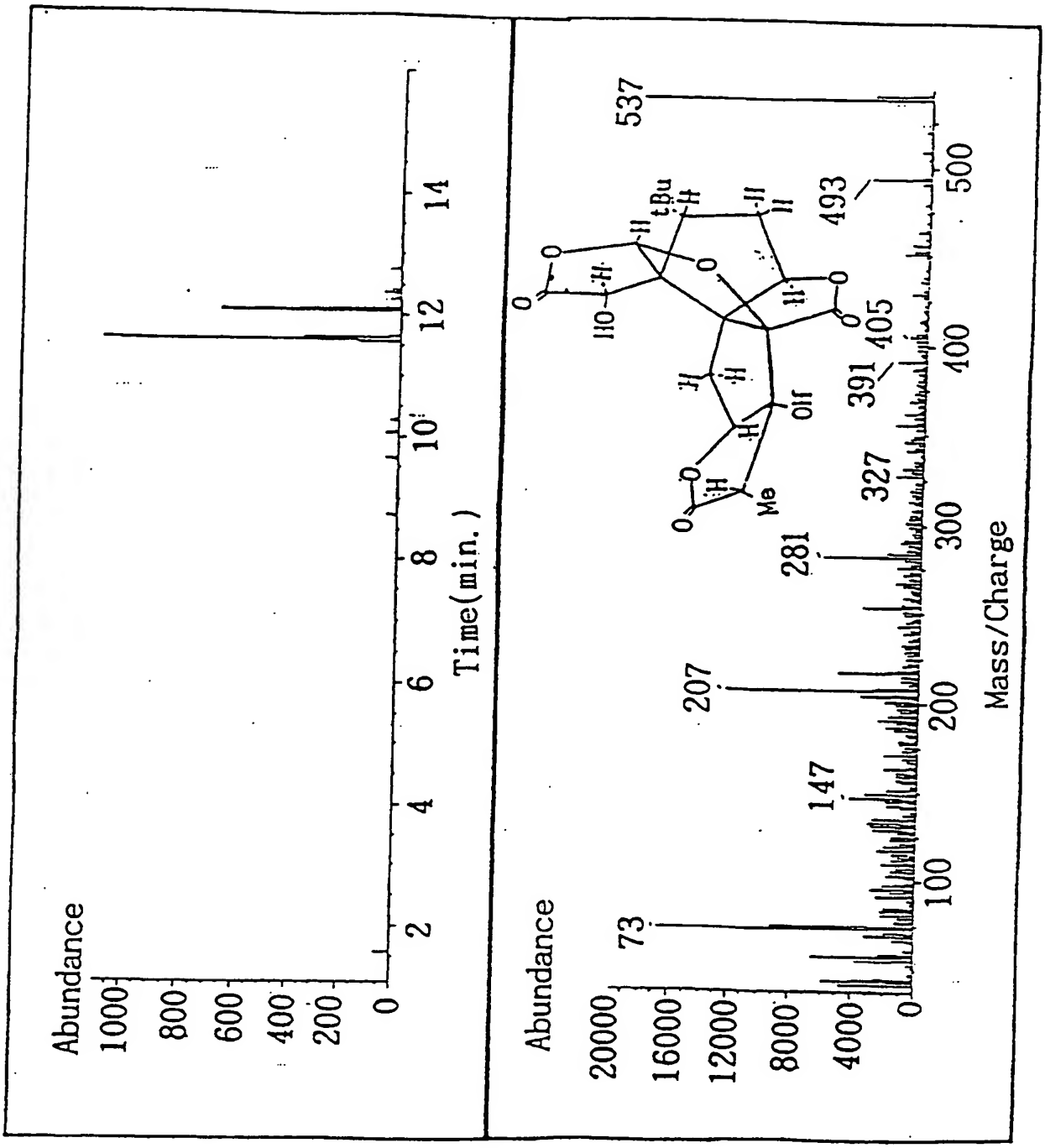


FIG.8-3B-a

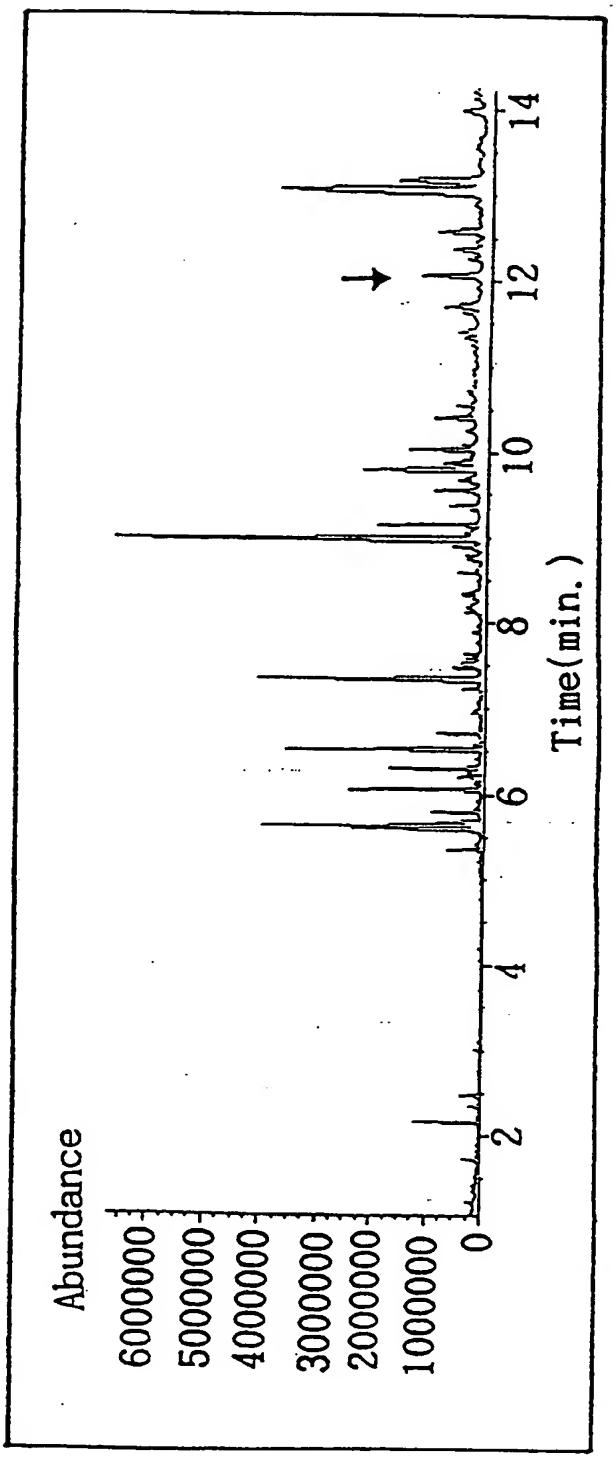

Abundance
6000000
5000000
4000000
3000000
2000000
1000000
0
2
4
6
8
10
12
14
Time(min.)

FIG.8-3B-b

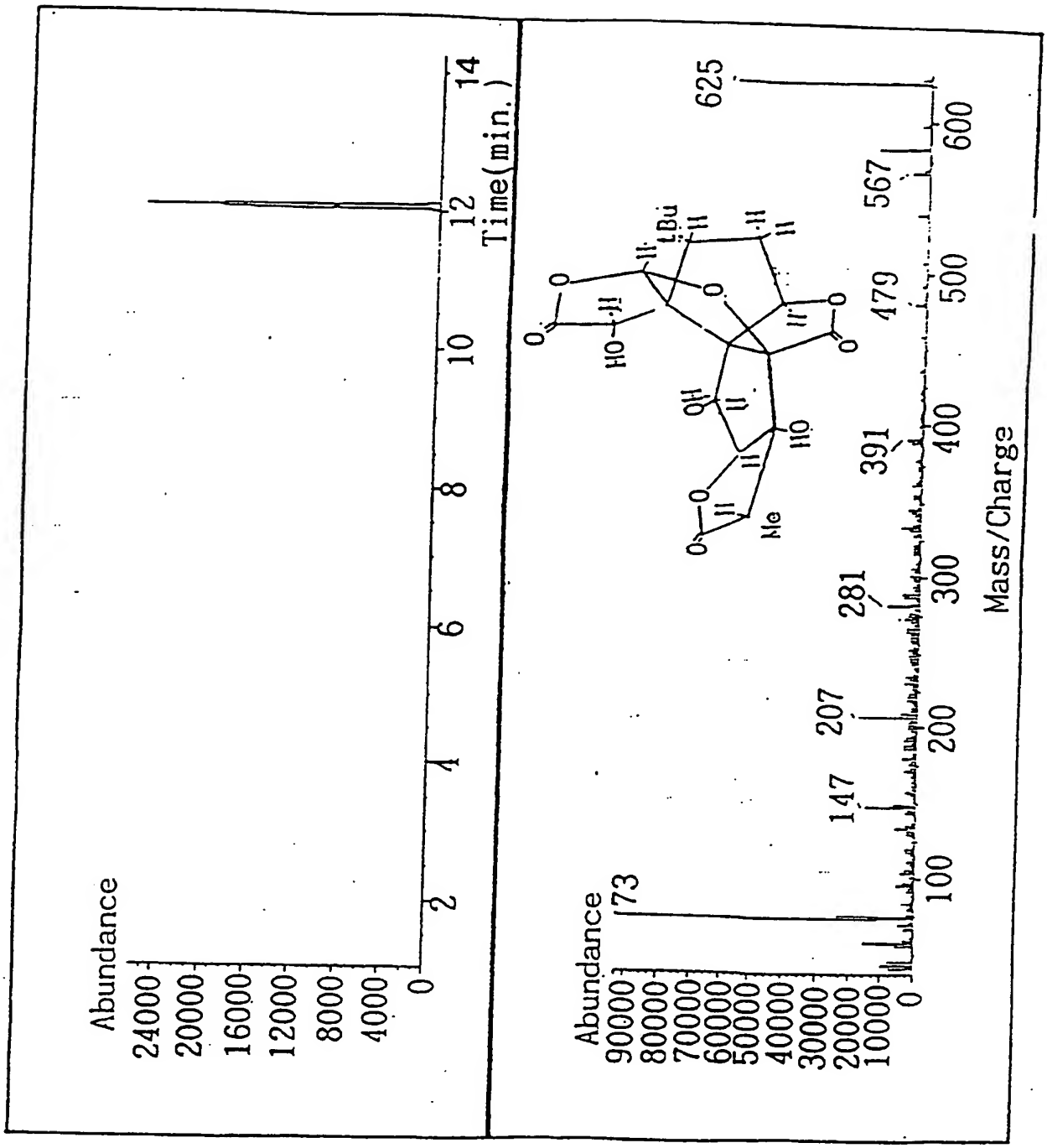


FIG.9-1

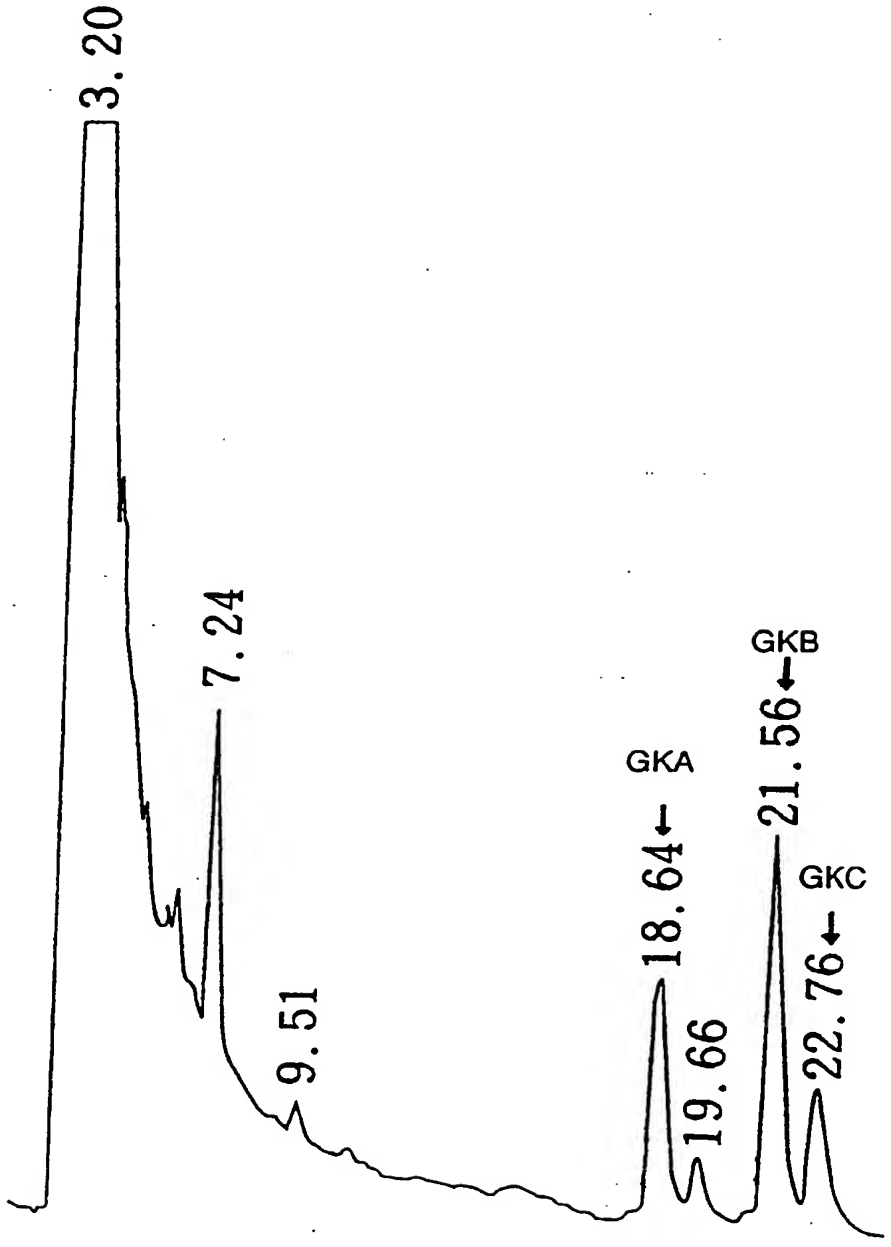


FIG.9-2

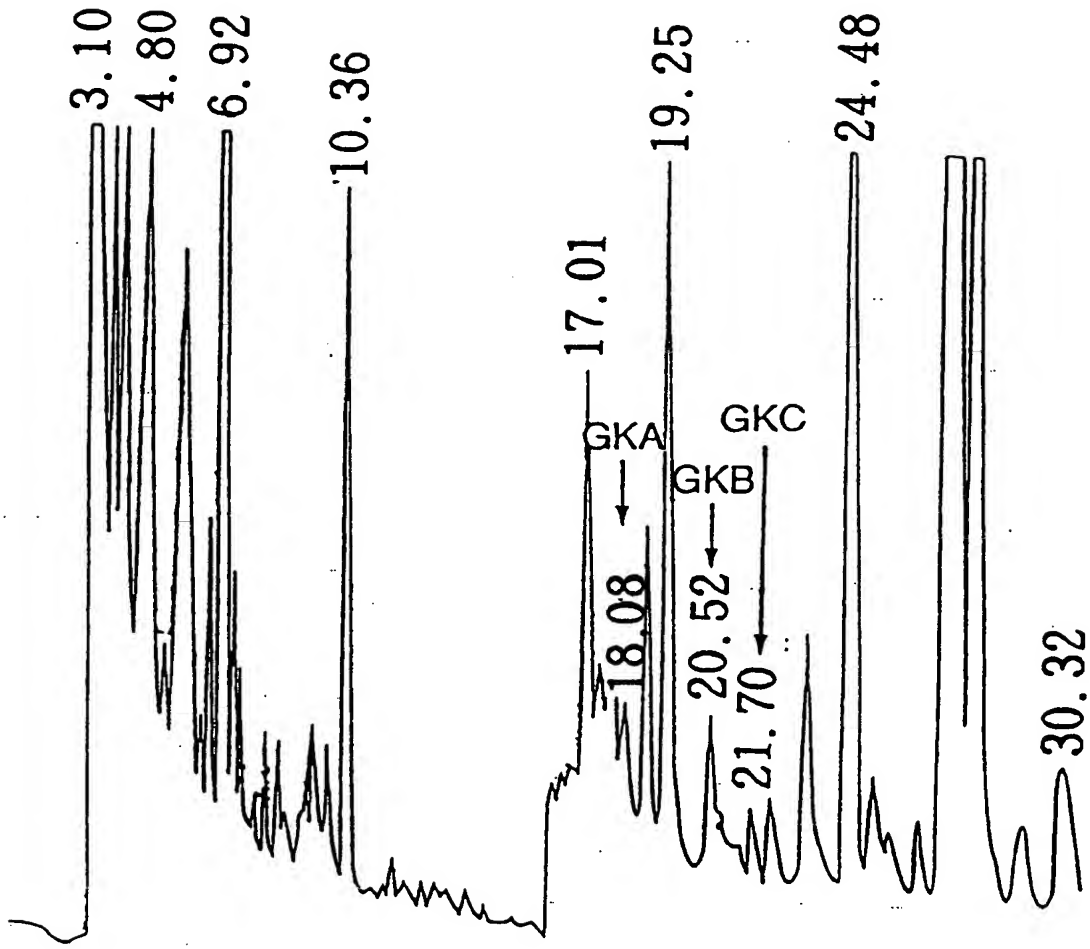

3.10
4.80
6.92
10.36
17.01
19.25
24.48
GKA
GKB
GKC
18.08
20.52
21.70
30.32

FIG.9-3A

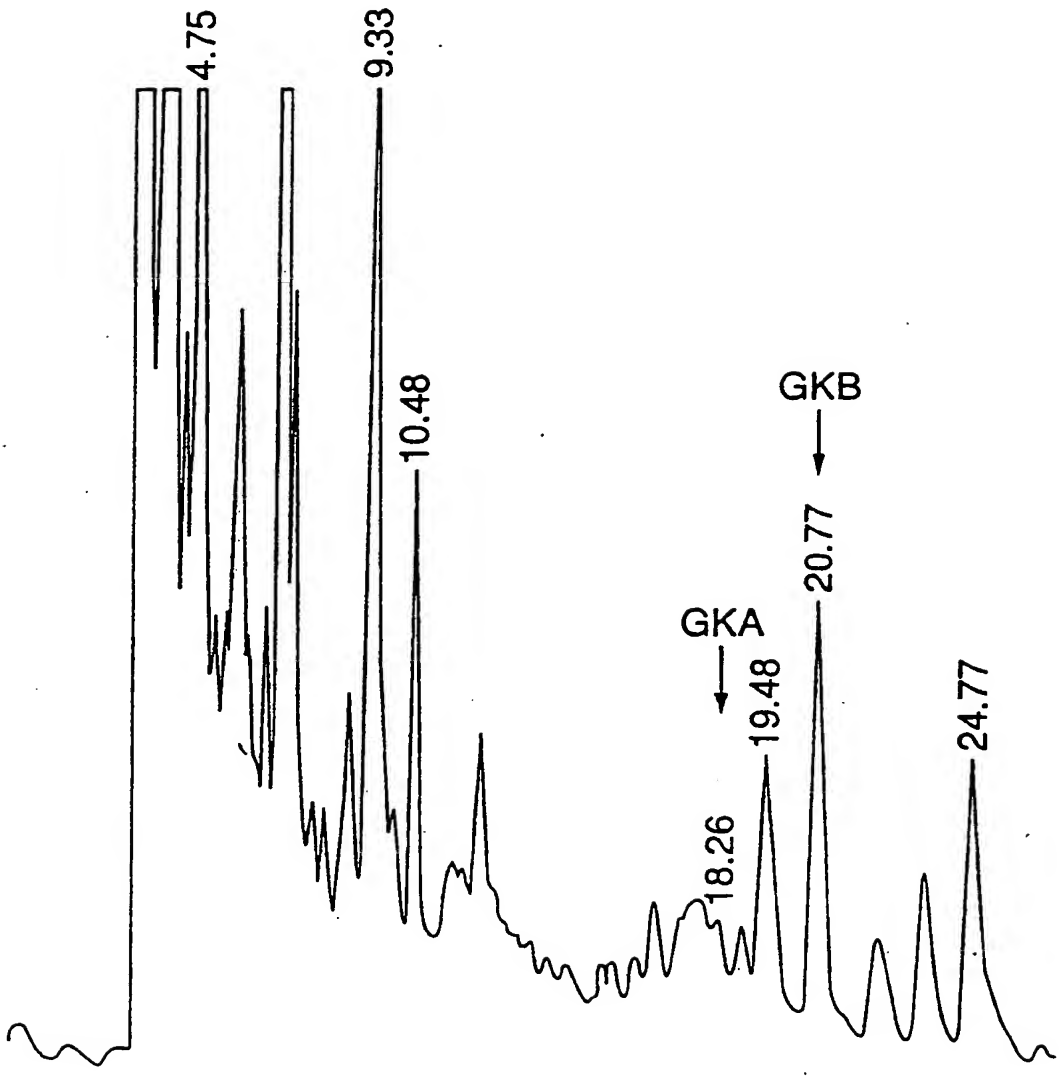


FIG.9-3B

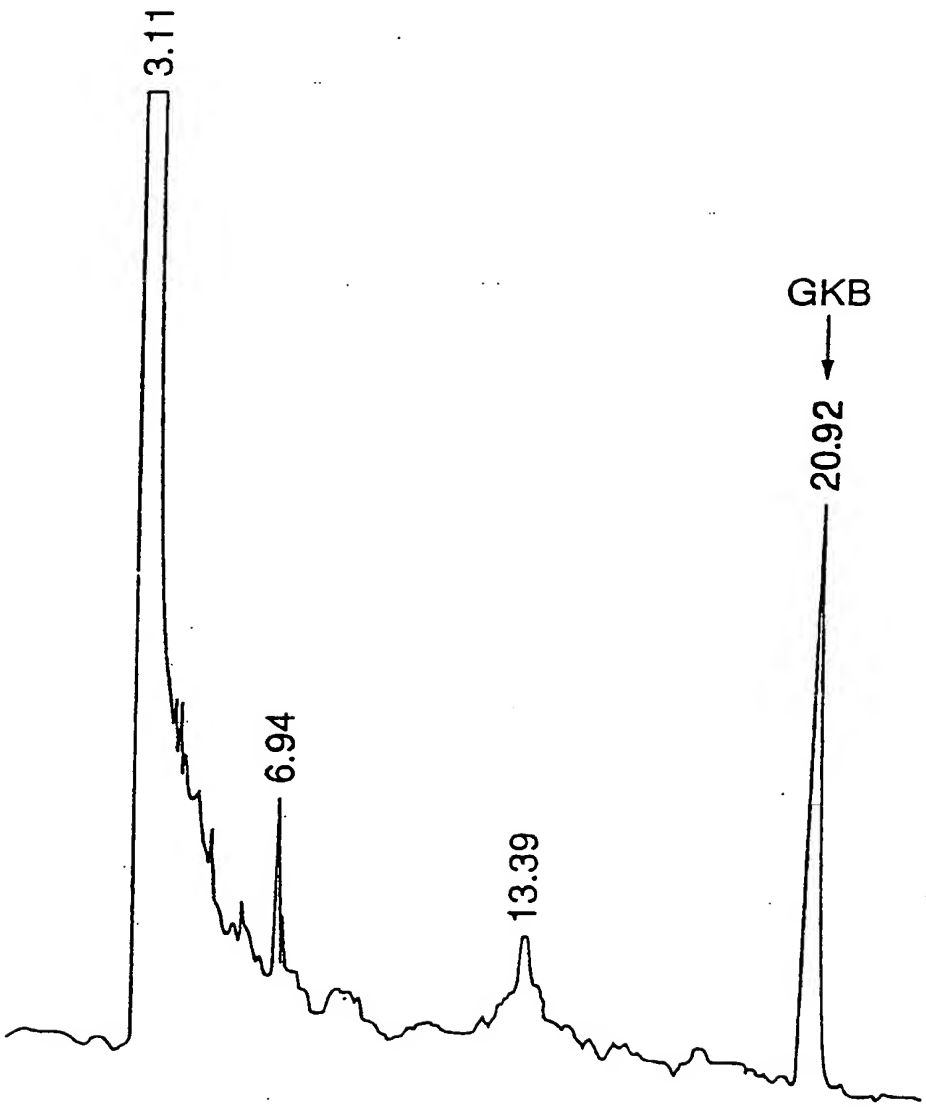
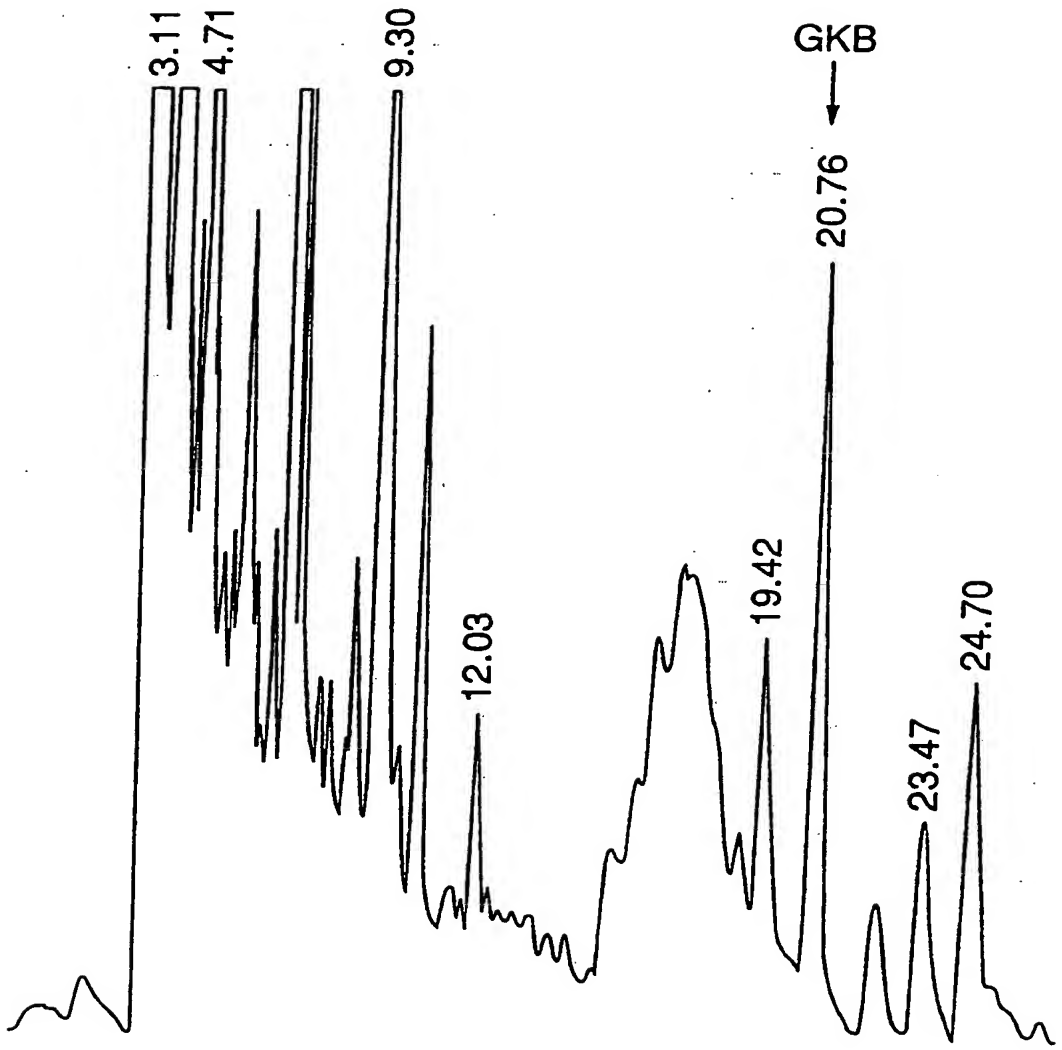


FIG.9-3C



LAST ADDED SHEET

SUBSTITUTE SHEET

FIG.9-4

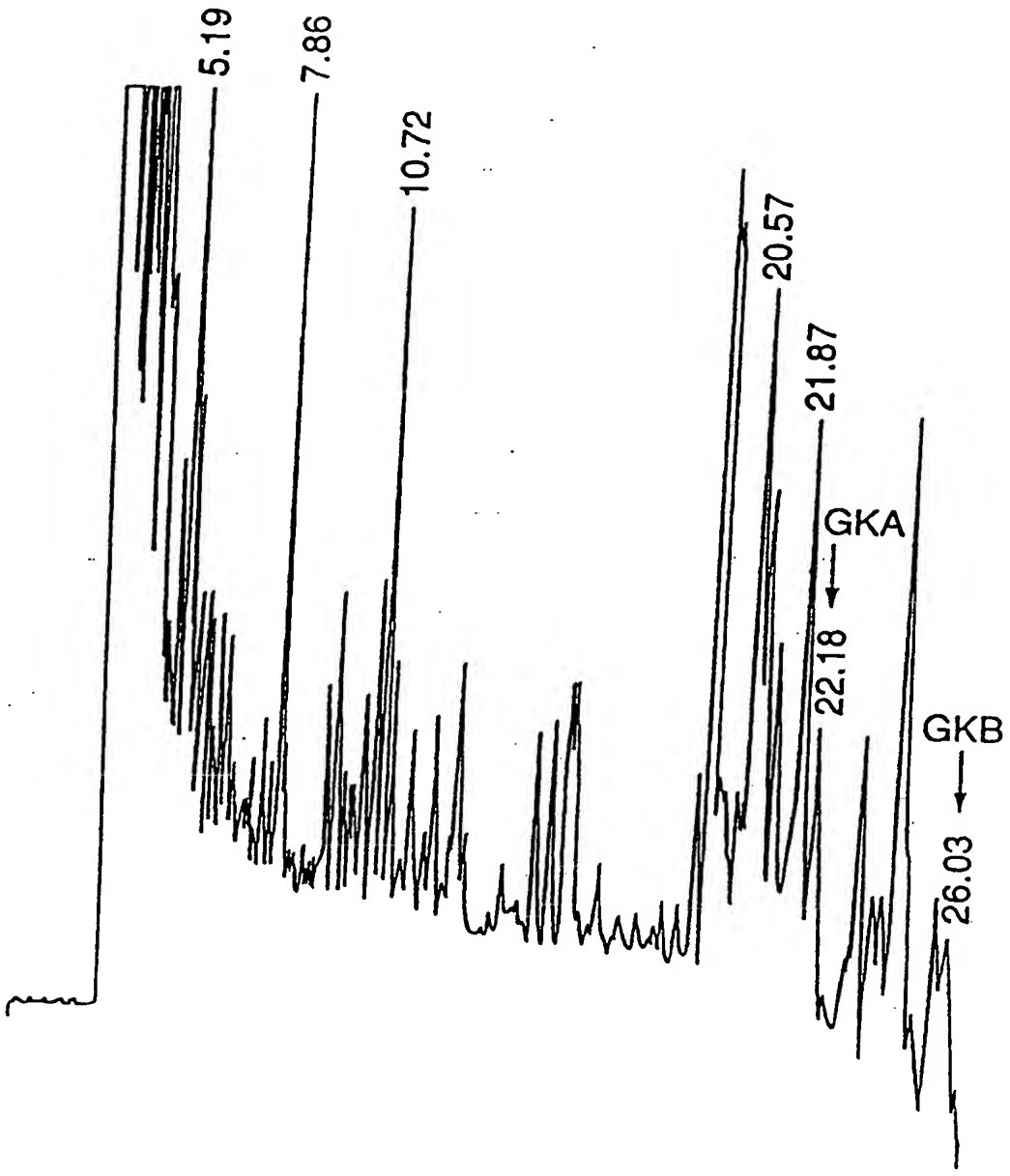


FIG.9-5

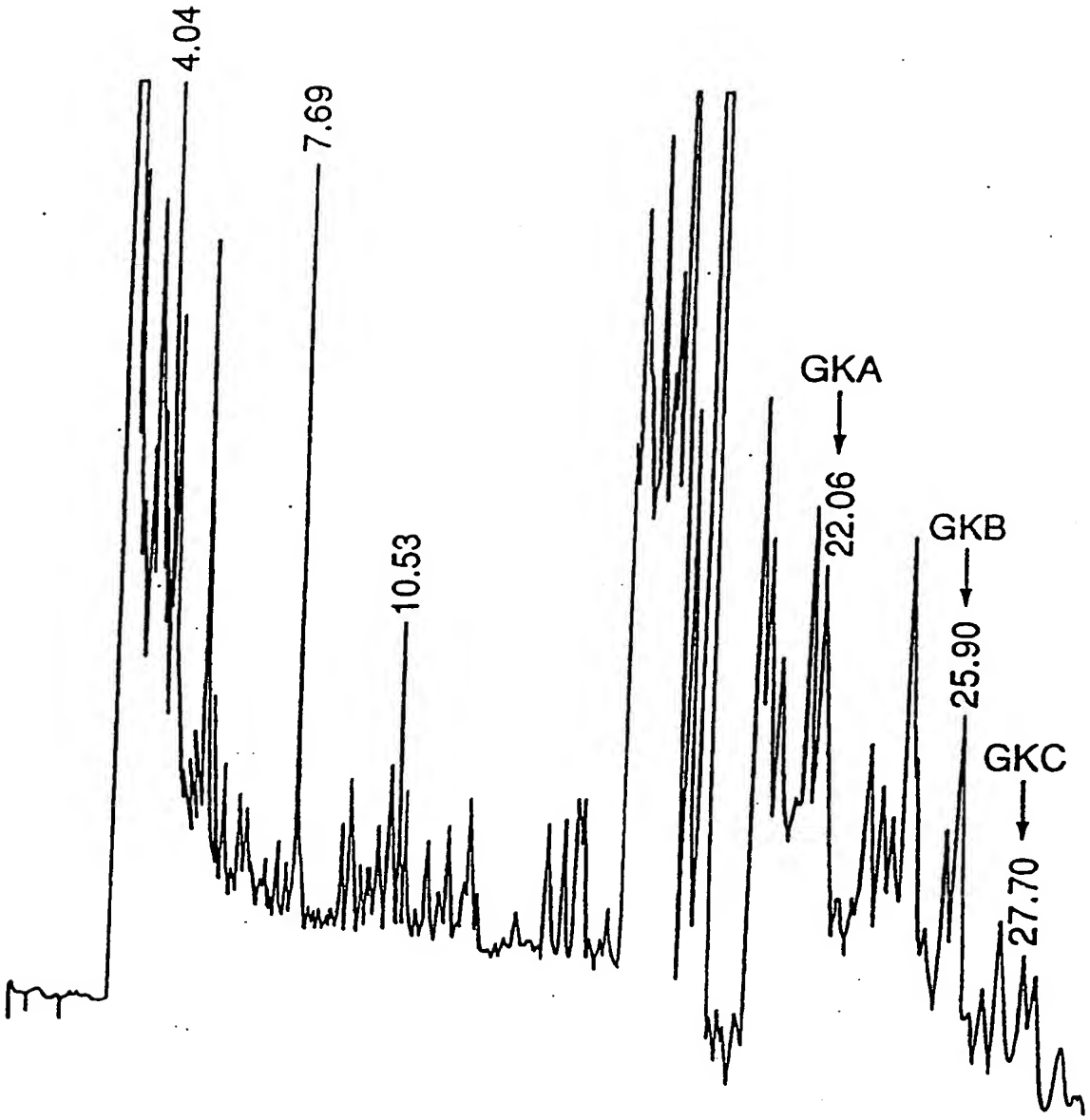
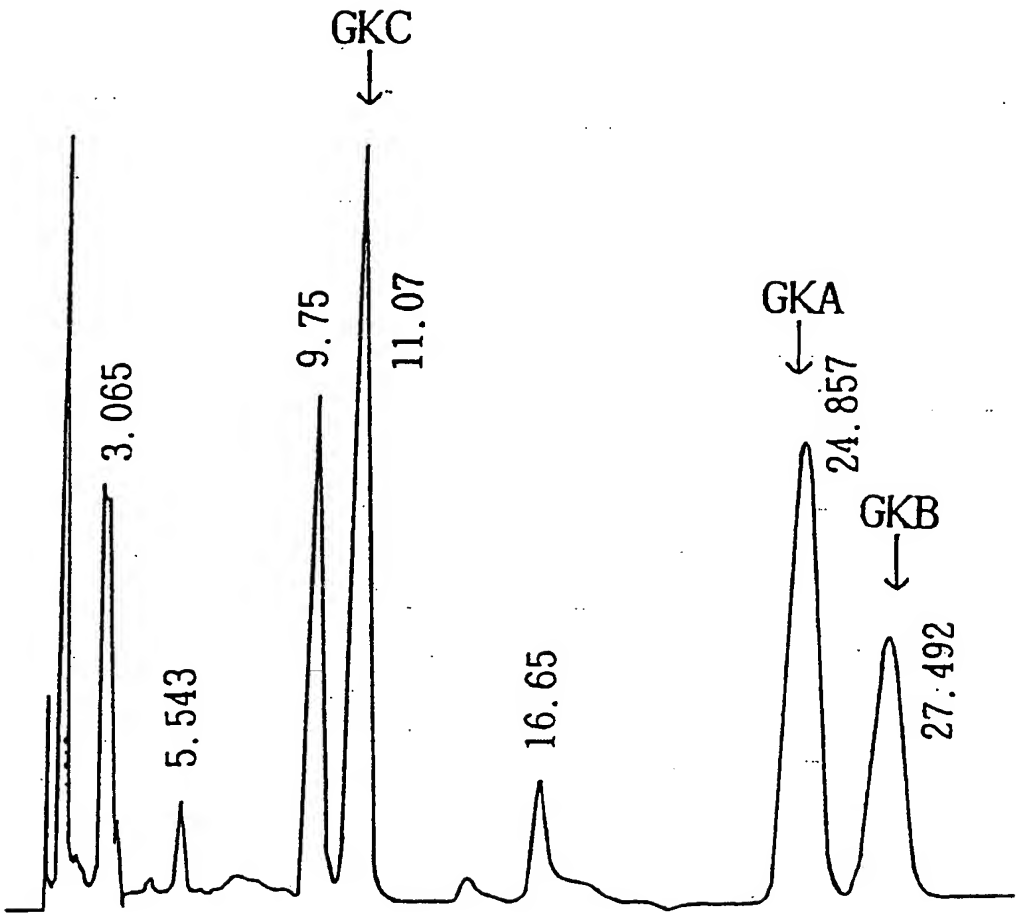
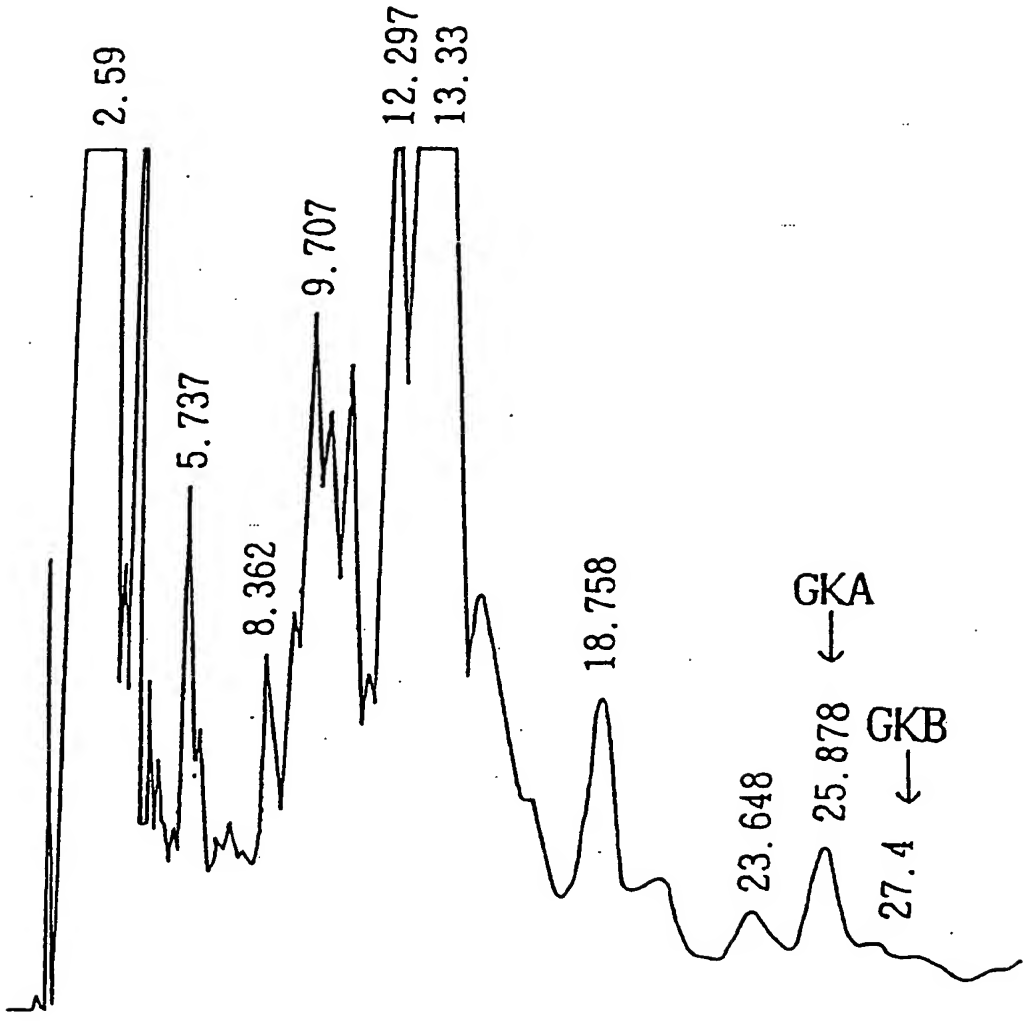


FIG.10-1



(29 TOTAL OF SHEETS)

FIG.10-2



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 92/00031

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl.[5]: C 12 P 17/02, A 61 K 35/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl.[5]: C 12 P 17/02, A 61 K 35/78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

WPIL, ginkgolide, Ginkgo biloba

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A2, 0 402 925 (SUNKYONG INDUSTRIES) 19 December 1990 (19.10.90), see abstract.	1
A	DE, A1, 3 940 092 (SCHWABE) 6 June 1991 (06.06.91), see claims 1-4.	1
A	EP, A1, 0 303 277 (OXO CHEMIE) 15 February 1989 (15.02.89), see abstract.	1

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

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"&" document member of the same patent family

Date of the actual completion of the international search
3 September 1992 (03.09.92)

Date of mailing of the international search report
8 September 1992 (08.09.92)

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR 92/00031

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
EP A2 402925	19-12-90	EP A3 402925	08-01-92
		JP A2 3024084	01-02-91
		US A 5089636	18-02-92
DE A1 3940092	06-06-91	CA AA 2031386	05-06-91
		DE C2 3940092	19-09-91
		EP A1 431536	12-06-91
		EP TD 431536	19-12-91
		JP A2 3279332	10-12-91
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		JP A2 1156923	20-06-89